A-183116

AAMRL-TR-87-020
NMRI-87-2

1986 TOXIC HAZARDS RESEARCH UNIT ANNUAL REPORT

WILLIAM E. HOUSTON, Ph.D.
RAYMOND S. KUTZMAN, Ph.D.
ROBERT L. CARPENTER, Ph.D.

NORTHROP SERVICES, INC. - ENVIRONMENTAL SCIENCES
101 WOODMAN DRIVE
SUITE 12
DAYTON, OH 45431

APRIL 1987

Approved for public release; distribution unlimited.

HARRY G. ARMSTRONG AEROSPACE MEDICAL RESEARCH LABORATORY
HUMAN SYSTEMS DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433

NOTICES

When U S Government drawings, specifications, or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data, is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from the Harry G. Armstrong Aerospace Medical Research Laboratory. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with Defense Technical Information Center should direct requests for copies of this report to:

Defense Technical Information Center
Cameron Station
Alexandria, Virginia 22314

TECHNICAL REVIEW AND APPROVAL

AAMRL-TR-87-020

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

BRUCE O. STUART, Ph.D.
Director, Toxic Hazards Division
Harry G. Armstrong Aerospace Medical Research Laboratory

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S) AAMRL-TR-87-020, NMRI-87-2
6a. NAME OF PERFORMING ORGANIZATION Northrop Services, Inc. — 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION AAMRL, Toxic Hazards Division
6c. ADDRESS (City, State and ZIP Code) 101 Woodman Drive, Suite 12 Dayton, Ohio 45431	7b. ADDRESS (City, State and ZIP Code) HSD, AFSC Wright-Patterson AFB, Ohio 45433
8a. NAME OF FUNDING/SPONSORING ORGANIZATION — 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER F33615-85-C-0532, End 01/15/91
8c. ADDRESS (City, State and ZIP Code)	10. SOURCE OF FUNDING NOS.

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT NO.
62202F	6302	00	01

11. TITLE (Include Security Classification) TOXIC HAZARDS RESEARCH UNIT ANNUAL REPORT: 1986

12. PERSONAL AUTHOR(S)
William E. Houston, Raymond S. Kutzman, Robert L. Carpenter

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Yr., Mo., Day)	15. PAGE COUNT
Annual	FROM 01/86 TO 10/86	87 04	194

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB. GR.	acetylcholinesterase, acute inhalation chamber, acute inhalation exposures,

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

This report has been prepared as a review of the research program of the Toxic Hazards Research Unit (THRU) for the time period of January 1986 to October 1986. During this first year of the contract work begun under the former contract was completed and new studies were initiated. Investigations were conducted on the dermal and ocular toxicity of surfactants and hydraulic fluids of interest to the United States military. Candidate materials, under consideration for novel new uses and for replacement of currently used materials have been investigated for toxicological characterization. These materials have included hydraulic fluids, lubricating oils, clothing materials, organometallic complexes, and decontamination agents. Continued interest by the military in the toxicological characteristics of fuels has been addressed with studies designed to establish

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION	
UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS ☐		
22a. NAME OF RESPONSIBLE INDIVIDUAL Harvey J. Clewell, III, Lt Col, USAF, BSC	22b. TELEPHONE NUMBER (Include Area Code) (513) 255-3916	22c. OFFICE SYMBOL AAMRL/TH

DD FORM 1473, 83 APR — EDITION OF 1 JAN 73 IS OBSOLETE.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

18.

acute toxicity, airway reactivity, alpha-2u-globulin, behavior, blood chemistry, chloropentafluorobenzene, cholinesterase, chromatography, chronic, colorimeter, cytotoxicity, dermal distribution, diisopropylfluorophosphate, exposure, eye, genotoxicity, Halocarbon oil, hemolysis, hepatocytes, human erythrocytes, hydraulic fluid, hydrazine, inhalation, inhalation chamber, intramembranous aggregates, irritation, isolated ventilated perfused lung, kidney tubules, log-linear model, lung diffusion, lung metabolism, methemoglobin, miotic effects, model, monomethylhydrazine, mutation, NMRI/TD, oral organophosphate, organotin, Otto Fuel II, Percol, perfusion, pharmacodynamic, pharmacokinetic, pharmacokinetic modeling, polyalphaolefin, polyethyleneglycol dinitrate, 1,2-propanediol nitrate, 1-propanone-methylhydrazone, prototype, pulmonary function, reactivation, sarin, sensitization, simulant, skin, soman, subchronic, teratogenesis, Thermionic Specific Detector, Toxic Hazards Research Unit, toxicant distribution, Triethylborane, 2,3,4-Trimethylpentane

19.

analytical methods for measuring methylhydrazine concentration in blood, the conduct of dermal exposures of rats to hydrazine, investigation of the teratogenic potential of Otto Fuel II, and the development of cell culture methods for comparison of liver and kidney cell metabolism of trimethylpentane.

A new research effort to develop computer-based pharmacodynamic models has been initiated. These mathematical models of biological systems have direct application to the toxicology testing and research programs at the THRU in the design of protocols and the minimization of experimental animal use.

Additional new areas of study have been under development during the first year of the contract. Investigations of the toxicity of chemicals of interest to the Air Force Installation Restoration Program have been initiated. These studies may be used to develop a battery of tests for materials found at sites of potential pollution. Also, the toxicity of selected materials associated with chemical defense programs has been addressed including: the evaluation of selected chemicals associated with the production of binary agents under development by the US Army; investigation of the toxicity of materials which show potential as simulant agents to be used in training exercises; and pharmacokinetic modeling of the toxicity of organophosphate chemicals.

PREFACE

This 23rd annual report of the Toxic Hazards Research Unit (THRU) concerns work performed by Northrop Services, Inc. – Environmental Sciences (NSI-ES) on behalf of the U.S. Air Force under Contract Number F33615-85-C-0532. This document constitutes the first report under the current contract and describes the accomplishments of the THRU from January 1986 through October 1986.

The current contract for operation of the Laboratory was initiated in 1986 under Project 6302, "Occupational and Environmental Toxic Hazards in Air Force Operations," Task 00, Toxic Hazards Research, Work Unit number 63020001. Lt Col H.J. Clewell served as the technical contract monitor for the Harry G. Armstrong Aerospace Medical Research Laboratory.

This is a cosponsored U.S. Air Force/U.S. Navy research effort. That portion of the work effort sponsored by the Naval Medical Research and Development Command was under the direction of CAPT David E. Uddin, MSC, USN, and identified as Navy Task Area Number MF58524001, "Chemical Hazards/Exposure Limits."

W.E. Houston, Ph.D., served as Program Manager for NSI-ES, THRU. Acknowledgement is made to the staff of NSI-ES, THRU, for their significant contributions and assistance in the preparation of this report. Partial support for this program was provided by the Naval Medical Research Institute, Toxicology Detachment, Wright-Patterson Air Force Base, OH.

TABLE OF CONTENTS

(continued)

TABLE OF CONTENTS

SECTION 1

SUMMARY

The research activity and support operations of the Toxic Hazards Research Unit (THRU) constitute a continuing program independent of contract years with several distinct research efforts (technical directives) in progress at the beginning and end of each annual reporting period. This year's research program was conducted on fuels, hydraulic fluids, lubricating oils, groundwater contaminants, decontaminating solutions, and other materials including surfactants, binary agent components, chemical simulants, and Navy watch cap material. The results or current status of these studies are discussed in detail in the body of this report.

This document represents the 23rd annual report of the Toxic Hazards Research Unit, and the first report submitted by Northrop Services, Inc. – Environmental Sciences (NSI-ES). The THRU is a research team that operates a dedicated inhalation toxicology laboratory and provides support activities to investigate the potentially toxic properties of chemicals and other compounds of interest to the U.S. Air Force, U.S. Navy, and other agencies of the U.S. Department of Defense. This research team is an interdisciplinary group of NSI-ES toxicologists, chemists, engineers, toxicokineticists, pulmonary physiologists, and biostatisticians. NSI-ES also provides services in animal histology, veterinary support, and quality assurance.

A major effort has been put forth this year to renovate, upgrade, and prepare for large-scale toxicology research. The research facilities used by the THRU consist of animal exposure chambers and supporting laboratories previously described by MacEwen (1965), Fairchild (1967), and Thomas (1965). The Rochester exposure chamber system in Building 433 was renovated and modified for acute studies; these modifications include the addition of scrubbers to the exhaust lines. One Thomas Dome had been modified to provide secondary containment for two Wahmann chambers. Other modifications to the Domes include installation of pneumatic hatch openers to increase personnel safety when entering and leaving through the air locks, and connection of chamber exhausts to scrubbers. A distribution study has characterized the spatial variation and distribution of light hydrocarbons in the Thomas Domes during inhalation exposure operations. Air Force construction and NSI-ES chamber installation in the new general toxicology laboratory are nearing completion.

In this first year of operation, toxicology research on fuels has focused on hydrazine and monomethylhydrazine (MMH). Hydrazine is potentially toxic by both inhalation and skin penetration. The inhalation hazard can be minimized by respiratory protection. However, respirators do not prevent dermal exposure. The skin penetration route is being studied using

chambers designed to eliminate inhalation exposure. Data from the skin exposure studies are being used in pharmacodynamic modeling to develop vapor threshold limit values for dermal exposures with respirators. This approach to analyzing combined skin and respiratory exposure routes is being applied to other hydrazine-based rocket fuels. A method has been developed to analyze MMH in blood, which is essential in quantitating the rate of skin penetration and can also be applied to the measurement of the dimethylhydrazines.

Toxicology results on hydraulic fluids indicate that four water-in-oil emulsion fluids currently being developed for the Submarine Damage Prevention Program exhibited neither death nor toxic responses in rats. All four of the compounds showed mild irritation to the conjunctival tissue of rabbits. Three formulations of synthetic polyalphaolefin, which are being developed as a substitute for petroleum-based hydraulic fluids, proved nontoxic in a variety of laboratory animals treated dermally or orally. However, certain formulations containing additives that produced characteristic infrared absorption spectra were toxic when tested by inhalation exposure.

Mild to moderate eye irritation, which resolved after insult, was observed following application of ACRYLOID 953 and TC9596A, surfactants used by the military. Skin irritation was not observed. This study was begun by the previous contractor to verify results reported by the materials manufacturer who found both eye and skin irritation. In acute toxicity studies of QL (a binary agent component) and some of its precursors, intermediates, and by-products, two of the process chemicals (KB and TEP) were found to produce skin irritation and inhalation toxicity in varying degrees. A toxicological evaluation of samples from one lot of U.S. Navy watch cap material that had been reported by plant workers to cause dermal, eye, and pulmonary irritation did not indicate signs of edema, erythema, or skin sensitization. Physical differences were observed between the fibers in the questionable lot and lots in which no irritation was reported; these observed differences led to a postulation that a fine lint caused by cutting and handling the suspect material may have caused the reported adverse effects. Toxicology studies with chloropentafluorobenzene, a chemical warfare simulant proposed for use by the Air Force, indicate that this material is a mild skin irritant that produces mild conjunctival irritation in laboratory animals. Neither skin sensitization nor inhalation threat could be demonstrated in these animals. Acute toxicity studies for several groundwater contaminants showed that 1,1-dichloroethane produced toxic effects when inhaled. The LC_{50} for this compound was found to be approximately 13,000 ppm.

A major emphasis in this research program is the development of biologically based computer models and an understanding of toxicant chemistry of biological systems. Developing and validating these models allows investigators to refine their understanding of actual experiments. These models

are used to predict pharmacokinetic behavior of test compounds. Information obtained can be used to guide design of actual laboratory work or to extrapolate laboratory results to new situations.

REFERENCES

MacEwen, J.D. 1965. Toxic Hazards Research Unit Design and Construction Phase. AMRL-TR-65-125 (AD624473). Wright-Patterson Air Force Base, OH: Aerospace Medical Research Laboratory.

Fairchild, E.J., II. 1967. Toxic Hazards Research Unit Annual Technical Report: 1967. AMRL-TR-67-137 (AD834723). Wright-Patterson Air Force Base, OH: Aerospace Medical Research Laboratory.

Thomas, A.A. 1965. Low ambient pressure environments and toxicity. *Am. Med. Assoc. Arch. Environ. Health* 11:316-322.

SECTION 2

INTRODUCTION

2.1 HISTORY OF THE THRU

Since 1963, the Toxic Hazards Research Unit (THRU) has been conducting a distinguished program to provide the U.S. Air Force with toxicology research support. The THRU program serves to identify and characterize toxic chemicals and materials to which Air Force personnel may be occupationally exposed. To utilize the unique capabilities and equipment offered by the THRU, the U.S. Navy, in 1976, established the Naval Medical Research Institute Toxicology Detachment (NMRI/TD) at Wright-Patterson Air Force Base to study the toxic effects of compounds and materials of interest to the Navy. This coordinated USAF-USN program provides scientific information from which risk assessment criteria are developed, exposure limits are set, and engineering designs are evaluated with regard to toxic chemical and material exposures.

Aerojet-General Corporation, Azusa, CA, operated the THRU from 1963 until 1968. During this period, extensive facility modifications culminated in the installation and operation of the Thomas Domes, large environmental chambers designed for continuous, uninterrupted, contaminant exposure at reduced atmospheric pressures. SysteMed Corporation, a subsidiary of EDP Technology, Inc., in Washington, DC, assumed operation of the THRU in 1968 and continued until 1972. Research efforts generated data useful in military or civil aircraft emergencies, community emergencies, and chronic industrial exposures.

In 1972 the THRU contract was awarded to the Department of Community and Environmental Medicine at the University of California Irvine (UCI). From that date until January 1986, UCI's research efforts focused on aircraft environment and chronic occupational health problems, as well as studies of the potential oncogenic properties of chemicals used in military and civilian activities.

2.2 NORTHROP SERVICES, INC. – ENVIRONMENTAL SCIENCES

Northrop Services, Inc. – Environmental Sciences (NSI-ES), a subsidiary of the Northrop Corporation in Los Angeles, CA, successfully competed for operation of the THRU and began activities in January 1986. NSI-ES has a comprehensive plan for executing the required research of the THRU. This includes a coordinated program of utilizing in-house research supplemented by off-site NSI-ES capabilities as well as subcontracting options with Battelle Columbus Laboratories, Experimental Pathology Laboratories, and five major universities. NSI-ES also has established a scientific advisory board and a comprehensive list of consultants to assist in evaluating and providing recommendations for the direction of research efforts.

2.3 RESEARCH EFFORT OBJECTIVES

NSI-ES supports USAF and USN programs by providing complete toxicologic evaluation of military compounds, including the capability to design valid scientific protocols, develop new analytical methods, and assess a wide range of biological end points utilizing state-of-the-art techniques in order to generate scientifically defensible data for the Armstrong Aerospace Medical Research Laboratory (AAMRL).

Major emphasis, modifications, and new directions include upgrading the facilities and equipment by implementing the design of new equipment for acute and short-term exposures. To provide the continuous monitoring and control desirable in modern toxicology studies, an extensive effort is under way to develop and implement computer-controlled data acquisition, monitoring, and control. A new approach to toxicology research will be widely implemented by NSI-ES. A section within the toxicology program is devoted to physiologically based pharmacokinetic modeling. This modeling approach can be used to predict toxic agent levels in target tissues and thereby to develop more effective protocols. As a result of using models in the design of toxicology experiments, protocols, sampling, and sacrifice schedules can be developed for specific target tissues leading to the use of fewer experimental animals to generate the required study data. At the present time these modeling techniques are most applicable to volatile chemicals. Efforts will be made to model toxic agents with differing chemical characteristics.

2.4 TOXICOLOGY CONFERENCE

The planning and execution of an annual toxicology conference is required by the contract with the AAMRL. The first five conferences, beginning in 1965, were entitled "Conference on Atmospheric Contamination in Confined Spaces," and were designed to report on scientific programs to determine the toxic hazards that are generated in the atmosphere of aerospace systems and to develop tolerance limits to atmospheric contaminants for various mission durations.

The next 15 conferences were entitled "Conference on Environmental Toxicology." In 1970 the emphasis shifted from space cabin environments to general environmental problems in the workplace and investigation of fuels, propellants, and other health hazards associated with the new materials employed in Air Force operations. Many subjects, for example immunotoxicology, hydrocarbons, aerosol propellants, fire extinguishants, cellular toxicology, toxic effects of combustion and pyrolysis products, shale oil, hydrazine, and toxicokinetics have been covered in these conferences.

The 1986 conference, held on October 28-30, was designated the "16th Conference on Toxicology" to recognize the number of years that toxicology has been the central theme.

Each year papers have been presented by nationally prominent leaders in toxicology, and the most recent conference was no exception. Twenty-two papers were presented at the 16th Conference on Toxicology, the theme for which was "Predictive Toxicology." Featured were topics in both environmental and occupational toxicology. A poster session was also held. The conference is supported jointly by the Toxic Hazards Division of the AAMRL and the NMRI/TD.

SECTION 3

RESEARCH PROGRAMS

3.1 TOXICOLOGY OF CHEMICALS AND MATERIALS

3.1.1 Evaluation of the Eye and Dermal Irritation and Dermal Toxicity of ACRYLOID 953 and TC9596A

E.R. Kinkead and S.S. Henry

INTRODUCTION

ACRYLOID 953 and TC9596A are surfactants being considered for use by the U.S. Military. The material safety data sheets prepared by the manufacturers of these materials indicated that each material may cause mild to moderate eye and skin irritation. LD_{50} values for acute dermal toxicity are reported as >5 g/kg and >3 g/kg for ACRYLOID 953 and TC9596A, respectively.

To verify the manufacturers' results of mild to moderate irritation, eye and skin irritation tests were conducted. In addition, the dermal lethality of the materials was also measured.

MATERIALS AND METHODS

Test Agents

Both materials were supplied by the U.S. Air Force. Characteristics of the fluids are shown below in Table 3.1.1-1.

TABLE 3.1.1-1. TEST AGENT CHARACTERISTICS

	ACRYLOID 953	TC9596A
Manufacturer:	Rohm and Haas Co.	Texaco
Appearance and odor:	Clear, viscous liquid, sweet odor	Light red liquid, strong ammonia odor
Specific gravity:	0.90	0.91
Viscosity:	700-1150 Cst at 100°C	55 Cst at 100°C
Solubility in H_2O:	Negligible	Negligible
Vapor pressure:	<1 mmHg at 20°C	Low (no specific value reported)

Animals

The animals used in this study were male and female New Zealand White rabbits, weighing between 2 and 3 kg. A photoperiod of 12 h light (beginning at 0600 hours) and 12 h darkness was employed. The rabbits had food and water *ad libitum* and were caged in conformance with Institute of Laboratory Animal Resources (ILAR) standards for laboratory animal care.

Evaluation of Irritation Response

Evaluations of skin and ocular lesions were based on the scoring system of Draize (1959).

Eye Irritation

One-tenth of a milliliter of test material was applied to one eye of each of nine albino rabbits; the opposite eye was left untreated and served as a control. The eyes of the test animals were examined with fluorescein stain prior to use to ensure absence of lesions or injury. A topical anesthetic was instilled in the treated and control eyes of all rabbits prior to application of the test substance. The treated eyes of six rabbits remained unwashed. The remaining three rabbits received test material and then had the treated eye flushed for 1 min with lukewarm water starting no sooner than 20 to 30 s after instillation. Examination for gross signs of eye irritation was made at 4, 24, 48, 72, and 96 h and at 7 days following application. Scoring of irritative effects was based on the method of Draize (1959) in which the total score for the eye is the sum of all scores obtained for the cornea, iris, and conjunctiva.

Skin Irritation

A patch test was utilized to determine the degree of primary skin irritation of intact skin of albino rabbits. All hair was clipped from the backs and flanks of six rabbits 24 h prior to exposure to allow for recovery of the skin from any abrasion resulting from the clipping. Each of the six rabbits was treated simultaneously with both test agents applied to specific, unique areas. Undiluted test material (0.5 mL) was applied to the designated patch areas and covered by a 1-in. square of surgical gauze, two single layers thick. The gauze patch was held in place with strips of surgical adhesive tape. The entire area was covered with dental dam secured with surgical adhesive tape. After 4 h, the wrap and patches were carefully removed, excess material wiped from the skin, and the test areas evaluated for irritation using the Draize (1959) table as a reference standard. Irritation evaluations were also performed at 24, 48, and 72 h.

Acute Dermal Toxicity

Acute dermal toxicity was assessed in rabbits. Initially, five rabbits of each sex were dosed with 1.65 and 1.62 g/kg body weight of TC9596A and ACRYLOID 953, respectively. If toxicity became evident during the follow-up observation period, additional dose levels would be tested to establish

a 14-day LD_{50}. This dose would be calculated using the moving average interpolation method of Weil (1952).

Twenty-four hours prior to application of the test agent, the fur was clipped from the dorsal area and sides of each rabbit. The test agent was applied in equal amounts to both sides of the backs of five male and five female rabbits. The test compound was kept in place for 24 h by applying a 4" × 8" 8-ply gauze patch over the exposed area. The midsection of each rabbit was also wrapped with dental dam and Elastoplast® tape. Twenty-four hours after application of the test agent, the wrappings were removed, and excess agent was wiped from the rabbits.

Animals were observed hourly during the day of dosing and twice daily during the 14-day observation period. Visible signs of toxicity were noted. Body weights of all animals were recorded at the time of dosing and on days 4, 7, 10, and 14.

RESULTS

Eye Irritation

ACRYLOID 953

Mild to moderate eye irritation was noted in all (nine out of nine) rabbits at the initial 4-h examination. Irritation of the conjunctivae was present in all unwashed rabbit eyes at 24 h. Two of the three washed eyes showed no irritation at 24 h. Mild conjunctival redness persisted in one rabbit throughout 48 h following dosing. Eye irritation scores at 72 and 96 h and at 7 days were all zero.

TC9596A

Mild to moderate eye irritation was recorded for eight of the nine rabbits at 4 h postdosing. At 24 h, four of the nine rabbits displayed redness of the conjunctivae; this redness persisted in one of the rabbits throughout the 48-h period following dosing. Signs of eye irritation were not noted at subsequent observations.

Skin Irritation

Both materials caused very mild erythema in two of six rabbits at 24 h. All signs of skin irritation had resolved by 72 h.

Acute Dermal Toxicity

Five male and five female rabbits were dosed with >1.60 g/kg of TC9596A. All rabbits appeared normal during the contact period and all gained weight during the 14-day observation period. Deaths did not occur as a result of 24-h skin contact to TC9596A.

Rabbits similarly dosed with ACRYLOID 953 appeared to gain weight normally during the 14-day observation period. However, 14 days after dermal exposure to ACRYLOID 953, one female rabbit died. Postmortem necropsy examination of the rabbit revealed no obvious cause of death; the single death, which occurred 14 days after dosing, appeared to be of extraneous rather than toxicity or dose-related causes.

DISCUSSION

The results of the eye and skin irritation tests conducted during this study indicate little irritation and no toxicity hazard through skin contact. Both fluids are considered mild to moderate eye irritants; the irritation resolves following insult. Neither skin irritation nor toxicity was evident as a result of contact with either fluid.

REFERENCES

Draize, J. H. 1959. Dermal Toxicity. In: The Staff of the Division of Pharmacology of the Federal Food and Drug Administration, eds. *Appraisal of the Safety of Chemicals in Food, Drugs, and Cosmetics*, pp. 46-59. Austin, TX: The Editorial Committee of the Associates of Food and Drug Officials of the United States.

U. S. Environmental Protection Agency. 1982. Office of Pesticides and Toxic Substances. Report No. EPA-560/6-82-001.

Weil, C. S. 1952. Tables for convenient calculation of median effective dose (LD_{50} or ED_{50}) and instructions in their use. *Biom.* 8:249-263.

3.1.2 The Evaluation of the Acute Toxicity of Four Water-In-Oil Emulsions Hydraulic Fluids

E.R. Kinkead, B.T. Culpepper, D.L. Pollard, and V.L. Harris

INTRODUCTION

For the Submarine Damage Prevention Program, the Navy was interested in evaluating four commercial water-in-oil emulsion fluids for use in high-pressure submarine internal hydraulic systems. The NMRI/TD requested that the THRU conduct a toxicological evaluation of these fluids upon which to base approval for shipboard use. The water-in-oil emulsion class of compounds consists of stable emulsions that contain 40% water as homogeneously dispersed particles of micronic or submicronic proportions suspended within a 60% continuous oil phase. The oil phase is formulated from a high-quality petroleum oil compounded with special additives to give the final product additional lubricity, corrosion protection, emulsion stability, and resistance to bacterial and fungal contamination.

The most significant exposure routes for hydraulic fluids are expected to be dermal (due to spills or leaks) and aerosol inhalation (from pressurized system leaks). The following studies reflect these potential routes of exposure. The studies included eye and skin irritation, skin sensitization, single-dose oral and dermal toxicity, and aerosol inhalation. Species and sex of animals selected for the acute toxicity tests are in conformance with the requirements of the Environmental Protection Agency (U.S. EPA, 1982).

The purpose of the studies was to develop data that provide a comparison of the short-term exposure effects of the four water-in-oil hydraulic fluids.

MATERIALS AND METHODS

Test Agents

A list of the four water-in-oil emulsions supplied by the NMRI/TD, including the various Navy codes is listed in Table 3.1.2-1.

Animals

Male and female Fischer 344 rats weighing between 150 and 250 g and between 125 and 250 g, respectively, male Hartley guinea pigs weighing between 300 and 650 g, and male and female New Zealand White rabbits weighing between 2 and 3 kg were used in these studies. All animals were shown to be in good health following a two-week quarantine period. Rats were housed, a maximum of four per cage, in clear plastic cages with wood chip bedding. The rabbits and guinea pigs were housed individually, the guinea pigs in plastic cages with wood chip bedding, and the

rabbits in wire-bottom stainless steel cages. Water and feed were available *ad libitum*, except when the rats were fasted for 16 h prior to oral dosing. Animal-room temperatures were maintained at 21 to 25°C, except for a two-day postinhalation exposure period when the building's heating system failed and the temperature dropped to 16°C. Some animals showed signs of stress from the temperature drop, such as shivering and diarrhea. The light/dark cycle was set for 12-h intervals.

TABLE 3.1.2-1 WATER-IN-OIL EMULSIONS

NMRI/TD No.	Supplier	Trade Name
86-049-1	Quaker Chemical Corp.	Quintolubric 958 30w
86-049-2	Mobil Oil Corp.	Pyrograud A-443
86-049-3	E.F. Houghton and Co.	Houghton-Safe 5047F
86-049-4	Sun Refining and Marketing Co.	Sunsafe F

	Pertinent Physical Properties			
	86-049-1	**86-049-2**	**86-049-3**	**86-049-4**
Boiling Pt. (°C):	100.00	100.00	106.70	100.00
Sp. Gravity (H_2O = 1):	0.96	0.92	0.92	0.92
% Volatiles by Vol.:	---	---	30-60	39.00
pH:	---	9.50	9.00	7.20
Appearance:	milky white fluid	milky white fluid	milky white fluid	milky white fluid

Evaluation of Response

The orally dosed rats that survived were weighed at 1, 2, 4, 7, 10, and 14 days postexposure, and any toxic signs observed were recorded during that period. On the 14th day postexposure gross pathology was performed. Rats surviving whole-body inhalation exposure were weighed on days 7, 10, and 14 postexposure. Any rat that died during or after inhalation exposure was subjected to gross pathology and histopathology. Surviving rats were subjected to gross pathology only. The sensitization testing procedure used on the guinea pigs was a modification of the Maguire (1973) method as described by Horton *et al.* (1981). Additionally, the guinea pigs were weighed on the first day of the testing period and on the final day before sacrificing. Evaluations of the rabbit skin and ocular lesions were based on the scoring system of Draize (1959). The rabbits' body weights were recorded on days 0, 1, 2, 4, 7, 10, and 14 of the dermal toxicity testing period.

Oral Toxicity

Sixteen hours prior to the administration of the oral dose, five male and five female F-344 rats, age seven to ten weeks, were deprived of food. Each rat was weighed and then given a 5g/kg dose by oral gavage at a volume of 0.01 mL/g body weight. Dilutions of the test materials were done with

corn oil that had been tested for the presence of peroxides. The peroxide level was not to exceed 3.0 meq/kg of corn oil. The corn oil was tested prior to use for dilution and revealed a peroxide level of 1.07 meq/kg, well within the limits set by our laboratory. Surviving rats were weighed at 1, 2, 4, 7, 10, and 14 days postexposure; toxic signs were recorded. An undosed control group was maintained for body weight measurements only. A repeated-measures test was used to compare body weights against controls (Barcikowski, 1983). Had deaths occurred at the limit test of 5g/kg, repetitions of this procedure using geometrically spaced doses were to be performed and the LD_{50} calculated using the moving average method of Weil (1952).

Dermal Toxicity

Twenty-four hours prior to dosing, the backs and sides of five male and five female New Zealand White rabbits weighing between 2 and 3 kg were clipped. The undiluted dose volume of 2.0 g/kg was applied to the back of the rabbits and spread evenly to both sides. The dose was kept in place by applying an 8-ply gauze patch over the liquid. A clear plastic wrap was then applied over the entire midsection and was held in place with Vetrap® and Elastoplast® tape. The dose was kept in contact with the rabbits' skin for 24 h. After 24 h, the tape, plastic wrap, and gauze were removed, and the residual test material was wiped from the animal. The animals' body weights were recorded on days 1, 2, 4, 7, 10, and 14 posttreatment. Toxic signs and mortality were monitored, and gross pathology was performed at the termination of the study. Had deaths occurred at the limit test of 2.0 g/kg, geometrically spaced doses would have been administered and the LD_{50} calculated using the moving average method of Weil (1952).

Inhalation Toxicity

Five male and five female F-344 rats, age seven to ten weeks, were placed in a 2.22-m^3 Rochester chamber with an effective volume of 1.45 m^3 and exposed for 4 h to the maximum achievable concentration of aerosolized test material. The limit test concentration of 5000 mg/m^3 could not be produced because of the physical properties of the test materials. The aerosol was generated by a Collison multijet nebulizer, and the aerosol concentration was gravimetrically analyzed during the exposure. Aerosol size measurements were conducted using an Andersen (Atlanta, GA) cascade impactor and a Lovelace (Albuquerque, NM) multijet impactor. Records were maintained for body weights (days 0, 7, 10, and 14 postexposure), toxic signs, and mortality. Gross pathology was performed on the day of sacrifice.

Eye Irritation

The eyes of nine New Zealand White rabbits of both sexes were examined with fluorescein stain prior to use to ensure absence of lesions or injury. The rabbits weighed between 2 and 3 kg. A topical anesthetic was instilled in the eyes, treated and control, of all rabbits approximately 2 min

prior to application of the test material. One-tenth of a milliliter of the test material was applied to one eye of each of the nine rabbits. The opposite eye was left untreated and served as the control. The treated eye of three rabbits was flushed with lukewarm deionized water for 1 min, starting 30 s after instillation. The eyes of the remaining six rabbits were not flushed. Examinations for gross signs of eye irritation were made at 4, 24, 48, and 72 h following dosing. Scoring of irritative effects was done according to the method of Draize (1944) where the total score for the eye is the sum of all scores obtained for the cornea, iris, and conjunctiva.

Skin Irritation

Six New Zealand White rabbits of both sexes were clipped on the backs and sides 24 h prior to dosing to allow for recovery of the skin from any abrasion resulting from the clipping. The test agent was applied in the amount of 0.5 mL to a designated patch area and was covered by a 3-cm^2 piece of surgical gauze, two single layers thick. The gauze patch was held in place with strips of surgical adhesive tape, and the entire shaved area covered with dental dam and secured with an elastic bandage and Elastoplast® tape. The patches remained in place for 4 h, at which time all wrapping was removed and the excess material wiped from the skin. The test areas were then evaluated for irritation using the Draize (1959) table. Additional evaluations were performed at 24, 48, and 72 h. The total score of the 4 observations for all rabbits was divided by 24 to yield a primary irritation rating, which was interpreted using the NIOSH (National Institute for Occupational Safety and Health) skin test rating (Campbell *et al.*, 1975).

Sensitization

Prior to the start of the study, three Hartley guinea pigs were clipped on both flanks and dosed with the test material so as to identify a nonirritating concentration to be used for the sensitization study. After the proper concentration was determined, 10 male guinea pigs to be used in the study were dosed with 0.1 mL of the diluted test material on the clipped left flank to determine the baseline irritation response. Additionally, 0.1 mL of the vehicle was applied to the clipped right flank of the same animal. Any hypersensitive guinea pig was eliminated from the study.

The site of sensitization used was an area immediately behind the shoulder girdle, which was clipped with an Oster® animal clipper and treated with a commercial depilatory 4 h prior to dosing. A Vetrap® frame with a 1.5 x 1.5 cm^2 opening at the site of the depilated area was affixed to each guinea pig. One-tenth of a milliliter of the test material was topically applied to the test area, and covered with gauze, dental dam, and Elastoplast® tape. This was done on Mondays, Wednesdays, and Fridays, until a total of four sensitizing doses were applied and evaluated. Along with the third sensitizing dose, 0.2 mL of a 50% aqueous dilution of Freund's adjuvant per animal was injected

intradermally using two or three sites next to the test site. Following the fourth sensitizing dose, the animals were rested for two weeks. Both flanks were then clipped and challenged on one flank with the test material and on the other flank with the vehicle. The challenge application was not occluded. Examination for signs of a sensitization reaction were made at 24 and 48 h. Any animal eliciting a score of 2 or more at the test solution challenge site at the 48-h scoring was rated a positive responder. The frequency of the reaction is the important statistic in determining sensitization potential. The information in Table 3.1.2-2 was used to classify the test materials as to sensitization potential.

TABLE 3.1.2-2. SCALE FOR DETERMINING SENSITIZATION POTENTIAL

Sensitization Rate (%)	Grade
10	Weak
20-30	Mild
40-60	Moderate
70-80	Strong
90-100	Extreme

RESULTS

Oral Toxicity

Forty F-344 rats (five males and five females per test material) were orally dosed. During the 14-day observation period all animals gained weight and showed no clinical signs of toxic response. No statistical difference was shown in body weight on day 0 (first day of study) or on day 14 (final day of study) between the control animals and the treatment groups. Statistical difference was calculated using the Multivariate Analysis of Covariance for Repeated Measures Test (Barcikowski, 1983).

Dermal Toxicity

Ten New Zealand White rabbits (five males and five females) were used per treatment group. No deaths resulted from the 24-h skin contact with the four test materials at a dose of 2 g/kg. All animals gained weight and showed no toxic signs over the 14-day posttreatment observation period.

Inhalation Toxicity

Ten F-344 rats (five males and five females) were used per test compound. No deaths occurred as a result of the inhalation exposure. All animals gained weight over the two-week postexposure period and showed no toxic signs. There was no statistical difference ($p < 0.05$) in percent gain in body weight between the controls and their corresponding treatment groups for males or females.

Analysis of the aerosolized water-in-oil emulsions showed the presence of ethylene glycol (thought to be the most toxic substance present in the test materials) to be well within the acceptable standards of a total weighted average of 125 mg/m^3 per 40-h work week (ACGIH, 1985). These data as well as the data on mass median aerodynamic diameter (MMAD), mean aerosol concentration, and percent nonvolatiles present are shown in Table 3.1.2-3. The aerosols of all four water-in-oil emulsions fell well within the respirable range.

TABLE 3.1.2-3. CHEMICAL ANALYSES OF FOUR WATER-IN-OIL EMULSIONS DURING 4-H INHALATION

Test Compound	Mean Concentration (mg/m^3)	MMAD[a] (m) ± S.G.D.[b]	Ethylene Glycol (mg/m^3)	Nonvolatiles (%)
86-049-1	180	2.00 ± 1.90	37	84
86-049-2	110	2.25 ± 1.78	29	79
86-049-3	210	2.15 ± 1.77	23	87
86-049-4	180	1.90 ± 2.00	11	75

[a] Mass median aerodynamic dynameter measured by Lovelace multijet impactor.
[b] Standard geometrical deviation.

Eye Irritation

Thirty-six animals were used (nine per test substance) in this facet of the study. Prescreening rabbit eyes with fluorescein stain prior to treatment showed mild to intense staining of the corneal epithelium in several animals. The eyes showing opacity were not used in the study; however, throughout the study, the eyes of the rabbits would show opacity on one evaluation and would be clear on another screening. A literature search revealed a paper by Kikkawa (1972) noting the same phenomenon. His explanation for this observation was that it was the normal desquamation of the corneal epithelium and not a result of instillation of the test compounds. It is our belief that this also was the case with the rabbits in this study.

Mild conjunctival redness was present in two rabbits 1 h after dosing with compound 86-049-1. No redness was present at 24, 48, or 72 h, however. Six of the nine animals dosed with compound 86-049-2 showed mild conjunctival redness at the 1-h screening, and two continued to show redness at the 72-h evaluation. Seven of the nine animals dosed with compound 86-049-3 showed mild conjunctival redness at the 1-h screening; one of these had persistent redness at the 72-h evaluation. Of the animals dosed with compound 86-049-4, five out of nine showed mild conjunctival redness at the 1-h evaluation; none of these showed any redness at the 72-h screening. No animals showed any inflammation or irritation of the iris.

Skin Irritation

No rabbits from any treatment group showed signs of necrosis or edema at the 4-h observation. One of the six rabbits dosed with test compound 86-049-1 showed signs of erythema 4 h postexposure. Twenty-four hours after dosing, three animals showed signs of erythema and one showed signs of erythema and edema. At 48 h postexposure, three animals continued to show erythema, while at 72 h, two showed erythema. Of the rabbits treated with compound 86-049-2, one showed signs of erythema at the 24-h observation, while no others showed any signs of irritation at the other observation periods. No rabbits dosed with compound 86-049-3 showed signs of erythema or edema 4 h posttreatment; however, one rabbit displayed erythema at the 24-, 48-, and 72-h evaluations. Four hours posttreatment, one rabbit treated with compound 86-049-4 exhibited erythema, four rabbits at 24 h, one at 48 h, and two at the 72-h screening. Skin reactions were evaluated and scored according to Draize (1959) and interpreted according to the NIOSH interpretation of skin test ratings. These ratings revealed all four water-in-oil test compounds to be nonirritating.

The total score of the 4 observations for all rabbits was divided by 24 to yield a primary irritation rating (Table 3.1.2-4), which was interpreted using the NIOSH skin test rating.

TABLE 3.1.2-4. PRIMARY SKIN IRRITATION RESULTS IN THE RABBIT

Test Compound	Primary Irritation Index	Effect
86-049-1	0.46	Nonirritant
86-049-2	0.04	Nonirritant
86-049-3	0.13	Nonirritant
86-049-4	0.38	Nonirritant

Sensitization

Ten guinea pigs per test substance were dosed with the challenge application. A weak sensitization reaction was shown with test substance 86-049-1. No animals dosed with the challenge application of the other three test substances showed a sensitization response. Any guinea pig with a score of 2.0 or above was considered sensitized.

DISCUSSION

In the oral, dermal, and inhalation toxicity studies no deaths or toxic signs were observed with any of the four test compounds. All four compounds exhibited a mild irritating effect to the conjunctival tissue of rabbit eyes. No irritating effect was observed with any of the four test materials as a result of exposure to intact skin.

The skin sensitization test was designed to evaluate the potential of materials to act as antigens. Applications of small quantities of antigenic material over a period of time induces antibody production. The induction potential can then be evaluated by grading the response to a challenge administration of the material. The 48-h response of one guinea pig to test substance #6049-1 indicated the possibility of the material being a weak sensitizer. The remaining test compounds did not elicit a sensitization reaction.

Table 3.1.2-5 presents a summary of the test results of the four oil-in-water studies.

TABLE 3.1.2-5. COMPARISON OF ACUTE TEST RESULTS OF THE FOUR WATER-IN-OIL EMULSION FLUIDS

Test Compound	Oral LD_{50} (g/kg)	Dermal LD_{50} (g/kg)	Inhalation LC_{50} (mg/m^3)	Eye Irritation	Skin Irritation	Sensitization Test
86-049-1	>5[a]	>2[a]	>180[a]	mild	nonirritant (0.46)[b]	weak[c]
86-049-2	>5[a]	>2[a]	>110[a]	mild	nonirritant (0.04)[b]	negative[c]
86-049-3	>5[a]	>2[a]	>210[a]	mild	nonirritant (0.13)[b]	negative[c]
86-049-4	>5[a]	>2[a]	>180[a]	mild	nonirritant (0.38)[b]	negative[c]

[a] No deaths or toxic signs at these concentrations.
[b] Based on NIOSH skin test rating (0-0.9 = nonirritant).
[c] Test as 10% solution in mineral oil.

REFERENCES

Barcikowski, R.S. 1983. *Computer Packages and Research Design* Vol. 1, BMDP. Lanham, MD: University Press of America.

Campbell, K.I., E.L. George, L.L. Hale, J.F. Stara. 1975. Dermal irritancy of metal compounds. *Arch. Environ. Health.* 30:168-170.

Draize, J.H. 1959. Dermal toxicity. In: The Staff of the Division of Pharmacology of the Federal Food and Drug Administration, eds. *Appraisal of the Safety of Chemicals in Food, Drugs, and Cosmetics,* pp. 46-59. Austin, TX: The Editorial Committee of the Associates of Food and Drug Officials of the United States.

Draize. J.H., G. Woodard, and H.O. Calvery. 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J. Pharmacol. Exp. Ther.* 32:377-390.

Horton, J. R., J.D. MacEwen, E.H. Vernot, and A. Hall. 1981. Comparisons of skin sensitization methods: Landsteiner, Maguire and guinea pig maximization. AFAMRL-TR-81-131. Wright-Patterson Air Force Base, OH.

Kikkawa, Y. 1972. Normal corneal staining with fluorescein. *Exp. Eye Res.* 14:13-20.

Maguire, H.C. 1973. The bioassay of contact allergens in the guinea pig. *J. Soc. Cosmet. Chem.* 24:151-162.

The American Conference of Governmental Industrial Hygienists (ACGIH). 1985. Threshold limit values and biological exposure indices for 1985-1986. Report No. ISBN: 0-936712-61-9. Cincinnati, OH.

U.S. Environmental Protection Agency. 1982. *Health Effects Test Guidelines.* Report No. EPA 560/6-82-001. Washington, DC: Office of Pesticides and Toxic Substances.

Weil, C.S. 1952. Tables for convenient calculation of median effective dose (LD_{50} or ED_{50}) and instructions in their use. *Biom.* 8:249-263.3.

3.1.3 Evaluation of the Acute Toxicity of a Synthetic Polyalphaolefin-based Hydraulic Fluid

E. R. Kinkead, S. S. Henry, H. F. Leahy, and C. Doarn

INTRODUCTION

The U.S. Naval Ship Research and Development Center is attempting to provide suitable replacement hydraulic fluids in the event that critical shortages of petroleum-based products should occur. A shortage of these fluids and lubricants would seriously affect the operational readiness of Navy ships. The Naval Ship Research and Development Center has developed some synthetic, polyalphaolefin-based materials that are intended to replace the petroleum oil-based hydraulic fluids described by the following military specifications: MIL-H-17672C, MIL-L-17331G, and MIL-F-17111A.

Five of the synthetic hydraulic fluids plus the polyalphaolefin stock were previously evaluated for their acute toxicological potential at the THRU, and a report of these tests can be found in the 1983 THRU Annual Technical Report (MacEwen and Vernot, 1983). Of the six fluids tested, only the one designated N501 demonstrated significant acute toxicity. Hydraulic fluid N501 was irritating to both rabbit and guinea pig skin and had an LC_{50} of approximately 2 mg/L for a 4-h aerosol inhalation in both male and female rats.

The hydraulic fluid N501 has been reformulated and is now designated B85-174. The reformulated hydraulic fluid and the polyalphaolefin stock R-1061-3 have been retested by the same methods and routes of administration as the previous study.

The species and sex of animals selected for the acute toxicity tests are in conformance with the requirements of the U.S. Environmental Protection Agency (U.S. EPA, 1982). Presently, there are no acceptable *in vitro* alternatives to these types of tests.

MATERIALS

Test Agents

All test fluids were supplied by the NMRI/TD. The hydraulic fluid contains polyalphaolefin as the major ingredient and antioxidant, antiwear, and rubber-swell additives as minor components. Information on the exact composition of the B85-174 fluid is proprietary. A sample of the neat polyalphaolefin (R-1061-3) has been provided for comparative purposes. The Navy code designations are listed below (Table 3.1.3-1).

TABLE 3.1.3-1. CODE DESIGNATIONS FOR TEST AGENT

NMRI/TD NO.	DTNSRDC NO. [a]
86-072-2	B85-174
86-133-1	R-1061-3[b]

a David Taylor Naval Ship Research & Development Center.
b Polyalphaolefin stock of sample B85-174.

A Beckman Acculab 4 was used to obtain infrared spectra of the two materials shortly after receipt. B85-174 and R-1061-3 had similar spectra (Figures 3.1.3-1 and 3.1.3-2) except for an increased absorption of some peaks in the 8- to 9-μ region for B85-174, the polyalphaolefin containing additives. Additional infrared spectra were obtained just prior to use in the aerosol studies and also following generation of the test aerosol by sampling the fluids remaining in the generation system reservoir. Comparison of infrared spectra demonstrated no change during storage prior to toxicity testing, and the spectra from the recycled material remaining in the reservoir following inhalation exposures were similar to those of the starting materials.

It is significant that the stock polyalphaolefin fluid R-1061-3 received for use in this study was from a different supplier than the stock fluid used in 1983. An infrared spectrum was obtained of the archived base stock hydraulic fluid N448 used in the 1983 study. The infrared spectrum of N448 (Figure 3.1.3-3) is similar except for the complete lack of the peaks in both the 5.5- to 6-μ and 8- to 9-μ regions. These areas are representative of the presence of esters.

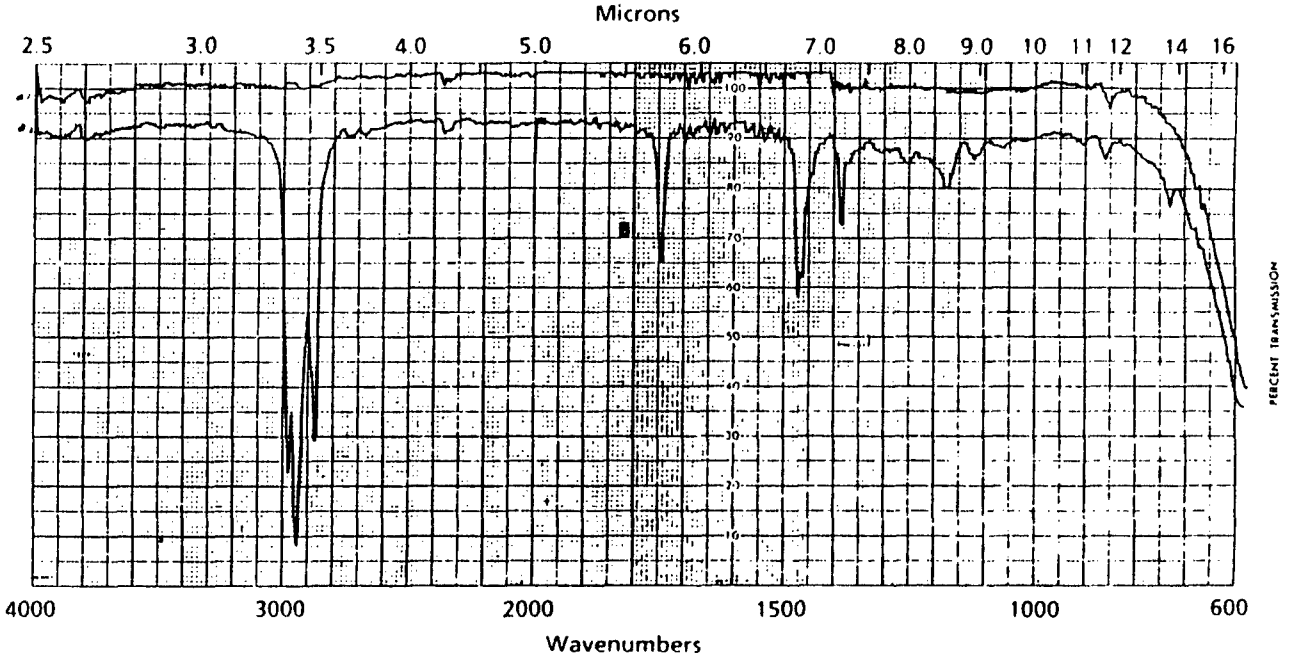


Figure 3.1.3-1. Infrared Spectrum of B85-174.

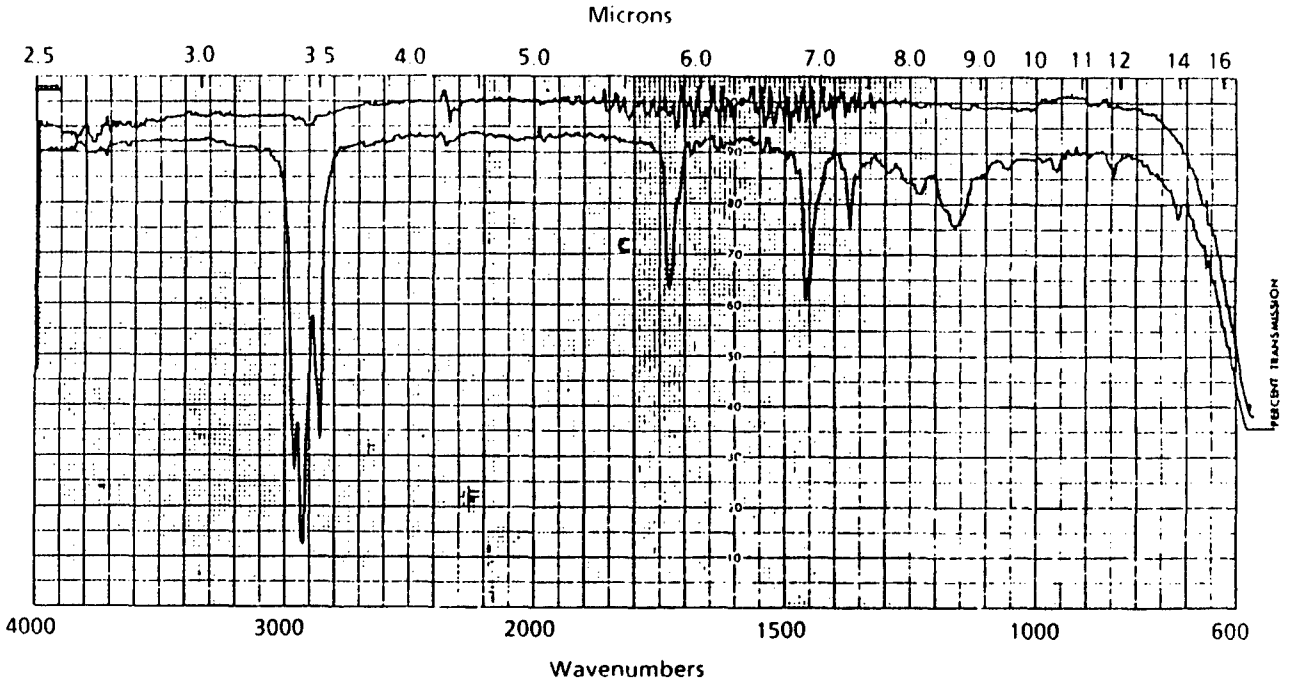


Figure 3.1.3-2. Infrared Spectrum of R-1061-3.

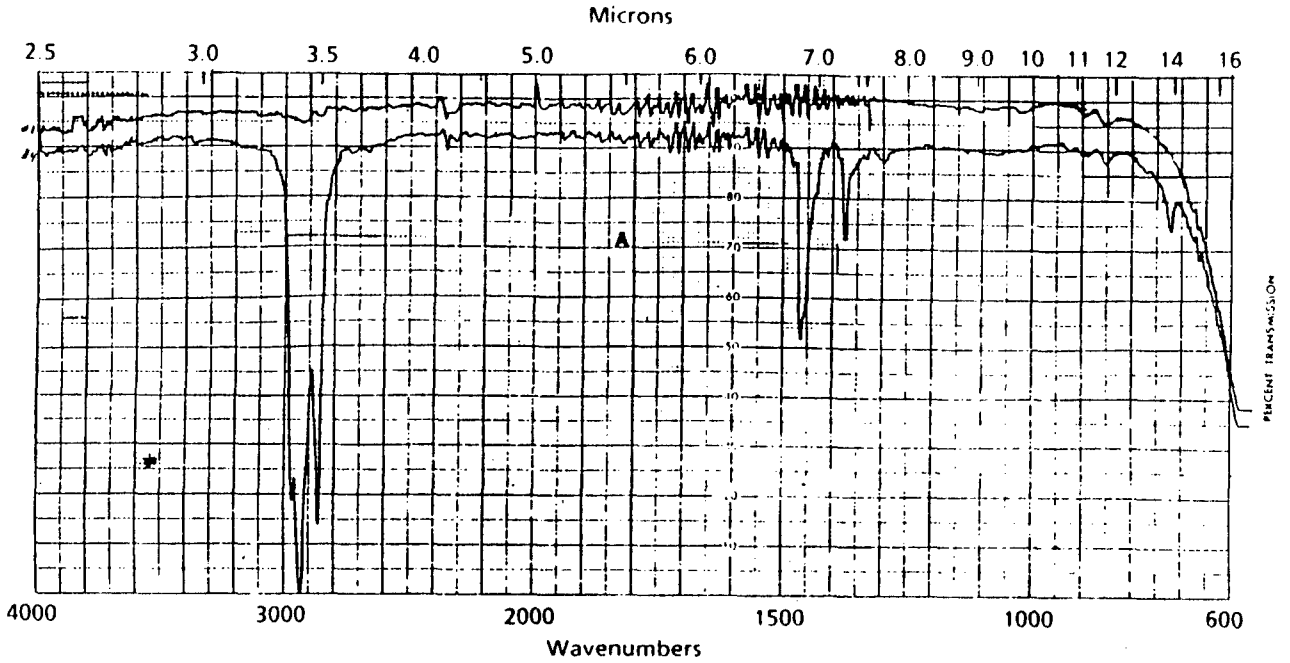


Figure 3.1.3-3. Infrared Spectrum of N448.

Animals

Male and female New Zealand White rabbits, weighing between 2 and 3 kg, male, albino Hartley guinea pigs, weighing between 300 and 650 g, and male and female, Sprague-Dawley rats, weighing between 200 and 300 g for males and 150 and 250 g for females, were used in this study. Quality-control determinations, made during a two-week quarantine period, showed the animals to be of acceptable health.

The rabbits and guinea pigs were housed individually, the guinea pigs in plastic cages with wood chip bedding, and the rabbits in wire-bottom stainless steel cages. Rats were group-housed with a maximum of four animals per cage, in plastic cages with wood chip bedding. Water and feed were available *ad libitum*, with the exception that rats were fasted for 16 h prior to oral dosing. The light/dark cycle was set at 12-h intervals. Ambient temperatures were maintained at 21 to 25°C except for one weekend when, because of a heating system failure, the room's temperature dropped to between 16 and 19°C. No study animals died during this period, and stress due to the lowered temperature was not apparent in the test results. Inhalation toxicity exposures, which were the only tests in progress at the time of the incident, were resumed 24 h after restabilization of the room's temperature.

Evaluation Scales

Evaluations of skin and ocular lesions were based on the scoring system of Draize (1959). The sensitization testing procedure is a modification of the Maguire (1973) method as described by Horton *et al.* (1981).

METHODS

Oral Toxicity

Five male and five female rats were deprived of food for 16 h and weighed prior to administration of the oral dose. The 5.0 g/kg limit test dose was administered by oral gavage. Each rat received a volume of 0.01 mL/g body weight, and the test materials were diluted with corn oil. Surviving rats were weighed at 1, 2, 4, 7, 10, and 14 days postexposure.

The corn oil used to dilute the test agents was analyzed for peroxide content two weeks prior to initiating this phase of the study and found to be well within the acceptable limit of 3 meq peroxide/kg corn oil.

Dermal Toxicity

The backs and sides of five male and five female rabbits were clipped 24 h prior to dosing. The proper dose volume (2.0 g/kg for the limit test) was then applied to the clipped area and spread

evenly. The dose was covered by 8-ply gauze squares, which in turn were covered by a clear plastic wrap surrounding the entire midsection of the body and, finally, by a layer of Elastoplast® tape. The test material was left in contact with the skin for 24 h, at which time the tape, plastic wrap, and gauze were removed, and residual material was wiped from the skin. Records were kept of body weights (at time of dosing and on days 1, 2, 4, 7, 10, and 14 posttreatment), toxic signs, and mortality.

Inhalation Toxicity

A stainless steel Wahmann Chamber was used to expose test animals to 4-h inhalations of the aerosolized test fluids. Various aerosol concentrations were produced with combinations of one, three, and six jet Collison nebulizers. Airflow through the chamber was equivalent to 12 to 15 air changes per hour. The chamber was operated at negative pressure. During the exposures, samples from the glass fiber filters and the Andersen (Atlanta, GA) cascade impactor were taken for gravimetric determination of chamber aerosol concentration and particle sizing, respectively.

Initially, five rats of each sex were exposed for 4 h to 5 mg test agent per liter of air (the limit test). Deaths did occur at the limit-test concentration of each test agent. Subsequent inhalation exposures utilized 10 rats of each sex for statistical purposes. Exposures were repeated at different concentrations until at least three dose levels, spaced appropriately to produce a range of toxic effects and mortality rates sufficient to permit the determination of an LC_{50}, were completed. A 14-day LC_{50} with 95% confidence limits was calculated using the probit analysis method of Finney (1971).

For all exposures the test animals were weighed prior to exposure and on days 7, 10, and 14 postexposure. Records of toxic signs and mortality were also kept. After mortality or sacrifice of animals, lung tissues were sampled for histopathologic examination.

Eye Irritation

For each fluid tested, the eyes of nine albino rabbits were examined with fluorescein stain to ensure absence of lesions or injury. A topical anesthetic was instilled in the treated and control eyes of all rabbits approximately 2 min prior to application of the test substance. One-tenth of a milliliter of test material was then applied to one eye of each of nine rabbits; the opposite eye was left untreated and served as a control. The treated eyes of six rabbits remained unwashed, whereas the remaining three rabbits received test material and then had the treated eye flushed for 1 min with lukewarm water, starting no sooner than 20 to 30 s after instillation. Examinations for gross signs of eye irritation were made at 4, 24, 48, and 72 h following application. Scoring of irritative effects was

based on the method of Draize (1959) in which the total score for the eye is the sum of all scores obtained for the cornea, iris, and conjunctiva.

Skin Irritation

A patch test was utilized to determine the degree of primary skin irritation of intact skin of albino rabbits. All hair was clipped from the backs and sides of six rabbits 24 h prior to exposure to allow for recovery of the skin from any abrasion resulting from the clipping. One-half milliliter of undiluted test material was applied to an approximately 3-cm^2 patch on the back of each rabbit. This was covered by 3 cm^2 of surgical gauze, two single layers thick. The patch was held in place with surgical adhesive tape, and the entire shaved area was covered with dental dam and secured with an elastic bandage and Elastoplast® tape. After 4 h, the wrap and patches were carefully removed, excess material was wiped from the skin, and the test areas were evaluated for irritation using the Draize (1959) table as a reference standard. Irritation evaluations were also performed at 24, 48, and 72 h.

Sensitization

Prior to the actual sensitization efforts, a separate group of three albino guinea pigs was used to determine a nonirritating dilution, if required, for use in the study. In addition, 0.1 mL of the diluted test material was applied to the clipped left flank of the study animals so that hypersensitive individuals could be identified and removed from the study.

The upper backs of ten guinea pigs were clipped and chemically depilated prior to the application of the first dose. The shoulder areas of the study animals were then wrapped with a latex bandage to outline the dosing area and to provide a base for holding cover bandages in place. One-tenth milliliter of test solution was then applied to a 1.5-cm^2 area and covered by gauze, dental dam, and Elastoplast® tape. This was repeated on Mondays, Wednesdays, and Fridays until a total of four sensitizing doses were applied. Along with the third sensitizing dose, 0.2 mL of a 50% aqueous dilution of Freund's adjuvant per animal was injected intradermally using two or three sites next to the test area. Following the fourth sensitizing dose, the animals were rested for two weeks.

Following two weeks of rest, both flanks were clipped and challenged, on one flank with 0.1 mL of test solution and on the other flank with 0.1 mL of the vehicle used to dilute the test material. The challenge applications were not occluded. The animals were examined for signs of a sensitization reaction at 24 and 48 h. Any animal eliciting a score of 2 or more at the test solution challenge site at the 48-h scoring was rated a positive responder. In scoring this test, the important statistic is the frequency of the reaction.

RESULTS

Oral Toxicity

Separate groups of five male and five female rats were dosed with 5 g/kg of B85-174 and R-1061-3. No deaths resulted from the oral administration of the test agents and no signs of toxicity were observed. All rats gained weight during the 14-day observation period.

Dermal Toxicity

Separate groups of five male and five female rabbits were dosed with 2 g/kg of B85-174 and R-1061-3. No deaths resulted from the 24-h skin contact with the test agents, and no toxic signs were observed. Nineteen of the twenty rabbits gained weight during the 14-day observation period, while one female rabbit maintained its weight.

Inhalation Toxicity

Separate groups of 10 male and 10 female rats were exposed to 5 mg/L aerosolized test agent B85-174 or R-1061-3, and 19 of those died within 48 h of exposure. Additional 4-h inhalation exposures were performed to determine LC_{50} values. The number of rats utilized in each exposure was increased to ten males and ten females for statistical purposes. Aerosol concentrations were raised or lowered in subsequent exposures based on previous mortality rates in order to obtain at least two partial mortalities and data near the total survival and/or total mortality ends of the range (Table 3.1.3-2).

TABLE 3.1.3-2. AEROSOL GENERATION AND RESULTING MORTALITY RATIOS IN 4-HOUR INHALATION EXPOSURES

	Aerosol Generation			Mortality Ratio (Deaths/Group Size)	
Test Agent	Conc.[a] ± std. dev. (mg/L)	MMAD[b]	GSD[c]	Males	Females
B85-174	5.03 ± 0.39	2.90	1.90	4/5	5/5
	2.50 ± 0.08	2.65	1.77	8/10	7/10
	1.57 ± 0.04	2.85	1.83	5/10	6/10
	0.88 ± 0.02	3.12	1.88	2/10	3/10
R-1061-3	4.96 ± 0.13	2.40	2.05	5/5	–
	5.03 ± 0.12	2.32	2.05	–	5/5
	1.30 ± 0.07	2.36	2.13	5/10	8/10
	0.70 ± 0.06	2.28	2.15	2/10	5/10
	0.50 ± 0.02	2.48	2.11	1/10	0/10

[a] Time-weighted average.
[b] Mass median aerodynamic diameter (micrometers).
[c] Geometric standard deviation.
– Not done.

Initial observations of the test animals during exposure revealed eye and nose irritation. Later, particularly at higher concentrations, the rats became inactive. Postexposure observations indicated labored, rapid, and shallow breathing; kyphosis; squinting eyes; lethargy; and bloody, nasal discharge. The rats' hair coats were also unkempt and wet with the test material.

Gross pathology of animals that died following exposure revealed congested, bright red colored lungs. Lungs, trachea, liver, and kidneys were sampled for histopathologic examination. The histopathologic studies are currently in progress, and a complete pathology report will be included in the final report.

No deaths occurred between the second and fourteenth (final) days of the observation period. Survivors gained weight during the second week of observation, and no toxic symptoms were observed during the second week postexposure.

LC_{50} values for B85-174 were determined to be 1.62 and 1.39 mg/L for males and females, respectively. For R-1061-3, the LC_{50} values were 1.23 and 0.85 mg/L for males and females, respectively (Table 3.1.3-3).

TABLE 3.1.3-3. ACUTE INHALATION TOXICITY LC_{50} VALUES

Test Material	Sex	LC_{50} (mg/L)	95% Confidence Limits (mg/L)	
			Lower	Upper
B85-174	Male	1.62	0.84	2.63
	Female	1.39	0.63	2.09
R-1061-3	Male	1.23	0.88	2.93
	Female	0.85	0.66	1.15

Eye Irritation

Fluids of test agents B85-174 and R-1061-3 were instilled in the eyes of separate groups of nine rabbits each. Neither test agent produced corneal opacity or congestion, or swelling or discharge of the iris or conjunctivae at 1, 24, 48, or 72 h postinstillation. This was true of whether or not the eyes were flushed with water after instillation of the test agent. All scores, individual and total, were zero.

Skin Irritation

Separate groups of six rabbits each were dermally exposed to test agents B85-174 and R-1061-3. No erythema, edema, or necrosis was observed in any rabbits following 4 h of dermal contact with the test agent. Subsequent observations at 24, 48, and 72 h were also negative in all respects.

Sensitization

Preliminary tests on albino guinea pigs demonstrated that, while both B85-174 and R-1061-3 produced erythema when applied neat, dilutions of 25% and 50% in mineral oil were nonirritating to the shaved flank skin. Based on these results, a 25% dilution of test material in mineral oil was used for sensitization testing.

Ten albino guinea pigs were used for each test material in the sensitization phase. Animals from the preliminary studies were specifically excluded from these groups. No test animals presented erythema or edema following application of the baseline response dose of 0.1 mL of 25% test solution to the shaved flank. Following 10 days of sensitization dosing and 2 weeks of rest, the test animals were again challenged with 0.1 mL of 25% test solution. The polyalphaolefin material R-1061-3 produced no erythema or edema at 24 or 48 h postdosing. The reformulated hydraulic fluid B85-174, however, proved to be a moderate sensitizer, producing erythematous reactions in 4 of the 10 test animals at 48 h postdosing.

DISCUSSION

The synthetic hydraulic fluid, B85-174, and its polyalphaolefin stock fluid, R-1061-3, were subjected to the same acute-toxicity, irritation, and sensitization tests as previously performed on five other formulations of polyalphaolefin-based hydraulic fluids and their stock fluids (MacEwen and Vernot, 1983). In the previous studies, the stock fluid and four of the five hydraulic fluids were found to be nontoxic. The N501 formulation, however, was mildly irritative to skin and toxic through inhalation exposure. B85-174 is a reformulation of the toxic N501. Comparison of the test results for B85-174 and N501 are given in Table 3.1.3-4.

TABLE 3.1.3-4. COMPARISON OF ACUTE TEST RESULTS FOR ORIGINAL (N501) AND REFORMULATED (B85-174) SYNTHETIC HYDRAULIC FLUIDS

Test Material	Eye Irritation	Skin Irritation	Sensitization	Oral LD_{50} (mg/kg)	Dermal LD_{50} (mL or g/kg)[a]	Inhalation LC_{50} (mg/L; 95% C.L.)
N501	Negative	Mild	Negative	>5	>2	2.39 (1.87-2.93)[b] 1.67 (0.95-2.23)[c]
B85-174	Negative	Negative	Moderate	>5	>2	1.62 (0.84-2.63)[b] 1.39 (0.63-2.09)[c]

[a] mL/kg for N501; g/kg for B85-174.
[b] Male rates.
[c] Female rats.

The calculated LC_{50} for the inhalation exposure to the reformulated hydraulic fluid (B85-174) is slightly lower than the previous N501 formulation. In other respects there are only minor

differences; N501 is a mild skin irritant, while B85-174 is a moderate sensitizer. With regard to eye irritation and oral and dermal toxicity, both are negative or nontoxic at the limit test value.

The current polyalphaolefin material is from Royal Lubricants Co., Inc., whereas the base material used in the 1983 studies was from Gulf Research and Development Company. Infrared spectra of these base materials are not the same (Figure 3.1.3-1). Tests on the polyalphaolefin material used in the 1983 studies (identified as N448) showed no toxicity, irritation, or sensitization. Current tests on the R-1061-3 material, however, reveal significant inhalation toxicity with LC_{50} values of 1.23 mg/L for male rats and 0.85 mg/L for female rats. In fact, these LC_{50} values are lower than those resulting from tests on the product B85-174 with additives. This suggests that the source of the toxicity in B85-174 is the base fluid from which it was made. This also raises the possibility that other synthetic hydraulic fluid formulations (such as the four that tested nontoxic in the 1983 studies) would be toxic via inhalation if compounded from the base fluid R-1061-3.

REFERENCES

Draize, J.H. 1959. Dermal Toxicity. In: The Staff of the Division of Pharmacology of the Federal Food and Drug Administration, eds. *Appraisal of the Safety of Chemicals in Food, Drugs and Cosmetics,* pp. 46-59. Austin, TX: The Editorial Committee of the Associates of Food and Drug Officials of the United States.

Draize, J.H., G. Woodard, and H.O. Calvery. 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J. Pharm. Exp. Therap.* 32:377-390.

Finney, D.J. 1971. *Probit Analysis.* New York, NY: Cambridge University Press.

Horton, J.R., J.D. MacEwen, E.H. Vernot and A. Hall. 1981. Comparisons of skin sensitization methods: Landsteiner, Maguire and guinea pig maximization. AFAMRL-TR-81-131 (ADA-110633). Wright-Patterson Air Force Base, OH: Air Force Aerospace Medical Research Laboratory.

MacEwen, J.D. and E.H. Vernot. 1983. The toxicologic evaluation of polyalphaolefin hydraulic fluids. In: Toxic Hazards Research Unit Annual Technical Report 1983. AFAMRL-TR-83-64 (ADA-136170), pp. 123-133. Wright-Patterson Air Force Base, OH: Air Force Aerospace Medical Research Laboratory.

Maguire, H.C. 1973. The bioassay of contact allergens in the guinea pig. *J. Soc. Cosmet. Chem.* 24:151-162.

U.S. Environmental Protection Agency. 1982. *Health Effects Test Guidelines.* Report No. EPA 560/6-82-001. Washington, DC: Office of Pesticides and Toxic Substances.

3.1.4 Protocol for the Determination of the Acute and Subchronic Oral Toxicity of Halocarbon Oil, Series 27-S

E. R. Kinkead

INTRODUCTION

In response to a request initiated by the Naval Sea Systems Command, the NMRI/TD has requested that the THRU investigate the acute and subchronic oral toxicity of Halocarbon Oil, Series 27-S (HC 27-S). The request was generated following contamination of a manned hyperbaric chamber with HC 27-S upon failure of a pump diaphragm.

The HC 27-S is a polymer of chlorotrifluoroethylene (CTFE), and is used as a lubricating oil for pumps employed in hyperbaric chambers. In addition, HC 27-S is used to lubricate all O-rings on doors and service locks of the chambers.

No specific toxicity information on the polymers of CTFE is available. Monomeric CTFE has been shown to produce renal damage in rats (Potter *et al.*, 1981; Buckley *et al.*, 1982). The principal insult in the kidney was necrosis of the pars recta, and to a lesser extent, the pars convoluta of the proximal tubule. Signs of nephrotoxicity included diuresis and increased urinary LDH activity, serum creatinine, and BUN, with a concurrent decrease in urine osmolality. Water intake increased 25% in exposed rats (Potter *et al.*, 1981). CTFE is metabolized to inorganic fluoride, which is excreted in the urine. In subchronic studies, tubules underwent regeneration and necrosis was minimal upon further exposure, suggesting adaptation to CTFE toxicity (Buckley *et al.*, 1982). The authors speculated that metabolism and/or disposition of CTFE was altered, or that regenerating tissue was refractive to CTFE insult.

These studies will be performed as a preliminary assessment of the toxicity of acute and subchronic exposure to HC 27-S, taking into consideration the toxicity reported for the parent monomer CTFE.

The rat has been selected as the test species to reduce space requirements and to allow for comparison to the above-mentioned CTFE studies. The number of animals per test group has been kept to the minimum necessary for appropriate statistical analysis. Existing alternative methods to animal testing are inadequate for this study.

MATERIALS

Test Material

The HC 27-S test material (Table 3.1.4-1) has been supplied by NMRI/TD.

TABLE 3.1.4-1. Test Material Data[a]

Item	HC 27-S
MILSPEC	MIL-L-24574
Federal Stock No.	9150-01-101-8835
Batch No.	86-75
Manufacturer	Halocarbon Products Corp., 82 Burlews Ct., Hackensack, NJ
Stability	Decomposes at temperatures above 260°C
Vapor Pressure	Less than 0.01 mmHg at 27°C
Additives	Organic acid rust inhibitor (0.1%)

[a] Data supplied by NMRI/TD.

Animals

Male and female Fischer-344 rats, weighing between 180 and 220 g and between 150 and 200 g, respectively, will be used in this study. The animals will be randomized according to a randomization regimen prepared by the NSI-ES Biometry Section, and toes will be clipped for identification.

Rats involved in the subchronic assay will be individually housed. During treatment they will be housed in Nalgene metabolism cages. Prior to the start of the study and during the 14-day holding period these rats will be housed in plastic cages with wood chip bedding. The rats employed in the acute assay will be group housed in plastic cages with wood chip bedding. Plastic cages will be changed twice per week and metabolism cages will be rinsed daily. All rats will be fed Purina Formulab 5008 (food in metabolism cages will be crushed) and softened water (not to exceed one grain per gallon hardness measured as calcium carbonate) *ad libitum*, with the exception that rats will be fasted for 16 h prior to oral dosing.

This study will be monitored by a veterinarian. Any animal determined to be in pain or distress will be euthanized. No anesthetics or drugs will be administered as their use would interfere with test results. All animals will be euthanized by CO_2 inhalation at scheduled sacrifice dates.

METHODS

Acute Assay (Oral LD_{50})

The rats will be fasted for at least 16 h prior to the administration of the oral dose. Initially, five male and five female Fischer-344 rats will be dosed with 5.0 g/kg. The rats will be individually weighed at the time of dosing, and the HC 27-S will be administered as a neat agent. The rats will be dosed one time and maintained for 14 days of observation.

Surviving rats will be weighed at 1, 4, 7, 10, and 14 days postexposure. The animals will be observed at least twice a day, and any toxic signs will be recorded on appropriate forms. At the end

of the 14-day observation period, all rats will be sacrificed. Gross pathology will be performed on all rats.

If deaths occur during the 5.0-g/kg limit test, additional studies will be performed by the same procedure. At least three dose levels will be used and spaced appropriately to produce a range of toxic effects and mortality rates sufficient to permit the determination of an LD_{50}. To reduce the numbers of animals required, geometrically spaced doses will be administered and the LD_{50} calculated using the moving average method of Weil (1952). Approximately fifty animals will be required for the acute assay.

Subchronic Assay

The subchronic study involves repeated dosing at one-half the LD_{50} concentration that has been determined in the acute study. If no deaths occur at the acute limit test, the dose level will be 2.5 g/kg. Six groups of Fischer-344 rats will be dosed daily, including weekends, by gavage. Each test group will receive the same dose, but the groups will be sacrificed at different times. Each control group will receive an equal volume of distilled water and will be sacrificed with its corresponding test group (Table 3.1.4-2). The HC 27-S will be administered as a neat agent. The volumes to be administered will be calculated from the individual body weights and adjusted weekly.

Dosing will be performed at the beginning of each day, prior to 0930 hours. Food will be provided following gavage and removed at 1630 hours each day. Initially, this study will be conducted using female rats, to be followed by an identical study using male rats. Approximately 100 animals will be required for the subchronic assay.

Body weights will be measured weekly throughout the study. Water consumption and urine output will be gravimetrically measured daily during the dosing period. Urine samples from one day predosing, from dose days 1, 3, 5, 7, 14 and 21, and from day 14 postdosing will be clinically analyzed according to Table 3.1.4-3.

TABLE 3.1.4-2. EXPERIMENTAL DESIGN[a]

Group	Dose Level	No. of Animals	Days on Test[b]	Days[b] at Sacrifice	Clinical Tests	Gross/ Histopathology
1	Control	6	7	8	yes	yes
2	$LD_{50}/2$[c]	6	7	8	yes	yes
3	Control	6	21	22	yes	yes
4	$LD_{50}/2$[c]	6	21	22	yes	yes
5	Control	6	21	36	yes	yes
6	$LD_{50}/2$[c]	6	21	36	yes	yes

[a] This design to be followed twice, first with all female rats then with all males.
[b] Consecutive days, including weekends.
[c] If no deaths occur at the limit test, the dose level will be 2.5 g/kg.

TABLE 3.1.4-3. URINALYSIS

Assay Method or Responsible Group	Tests
N-Multistix (Ames)	Protein Ketone Bilirubin Urobilinogen Hemoglobin pH Glucose
Air Force Personnel	Specific gravity Creatinine
Navy Personnel	Glutamic-oxalacetic transaminase (GOT) N-acetyl glucose aminidase (NAG) Lactic acid dehydrogenase (LDH) Calcium
NSI-ES, Chemistry Section	Inorganic fluoride[a]

[a] Fluoride specific electrode by method of Neefus *et al.* (1970).

The rats will be fasted 16 h prior to sacrifice. Groups 1 and 2 will be sacrificed on the morning following day 7 of dosing; Groups 3 and 4 will be sacrificed on the morning following day 21 of dosing; and Groups 5 and 6 will be sacrificed 14 days after the completion of dosing. At the time of sacrifice, blood will be collected from the posterior vena cava for whole-blood and serum analyses (Table 3.1.4-4). The following organs will be weighed: heart, pituitary, liver, spleen, thymus, kidneys, testes, ovaries and brain. A gross pathologic examination will be performed on each rat, and the following specified tissues will be collected and prepared for histopathologic examination.

nose	uterus	adrenals
bone marrow	jejunum	kidneys
parathyroid	stomach	prostate
heart	colon	ovaries
spleen	muscle (quadriceps, femoris)	ileum-duodenum
mandibular and mesenteric lymph nodes	bone (sternum and both femurs)	pancreas
urinary bladder	thyroid	brain
salivary glands	lungs	nerve (sciatic)
testes	liver	esophagus
seminal vesicles	thymus	pituitary
trachea		

TABLE 3.1.4-4. ANALYSIS OF BLOOD SAMPLES

Sample Type	Responsible Group and Assay method	Parameters
Whole blood	Air Force Personnel using Coulter counter	White blood cell count (WBC) with differential Red blood cell count (RBC) Hemoglobin Mean corpuscular volume (MCV) Mean corpuscular hemoglobin (MCH) Red cell distribution width (RDW) Hematocrit Platelet count Mean corpuscular hemoglobin concentration (MCHC)
Serum	Navy Personnel using Cobar Bio® centrifugal analyzer (Roche)	Glucose Creatinine Blood urea nitrogen (BUN) Alkaline phosphatase (AP) Creatinine phosphokinase (CPK) Serum glutamic-oxalacetic transaminase (SGOT) Serum glutamic-pyruvic transaminase (SGPT) Albumin Total protein Gamma glutamyl transferase (GT) Calcium
Serum	NSI-ES Chemistry Section using fluoride-specific electrode[a]	Inorganic fluoride

[a] Fluoride determination by method of Singer and Ophaug (1979).

Statistical handling of collected data will be as follows: A repeated multivariate analysis of variance with Scheffe pair-wise comparisons (Barcikowski, 1983) will be used for body weights, water consumption, urine volumes, and urine chemistries; a two-factorial analysis of variance with multiple comparisons will be performed for both blood parameters and organ weights (Barcikowski, 1983); and histopathologic data will be analyzed using either Fisher's Exact Test or, if not valid, Yates' corrected Chi-Square Test (Zar, 1974).

REFERENCES

Barcikowski, R.S. 1983. *Computer Packages and Research Design,* Vol. 1, BMDP. Lanham, MD: University Press of America.

Buckley, L.A., J.W. Clayton, R.B. Nagle, and A.J. Gandolfi. 1982. Chlorotrifluoroethylene nephrotoxicity in rats: A subacute study. *Fund. Appl. Toxicol.* 2:181-186.

Neefus, J. D., J. Cholak, and B.E. Saltzman. 1970. The determination of fluoride in urine using a fluoride-specific ion electrode. *Am. Ind. Hyg. Assoc. J.* 31:96-99.

Potter, C.L., A.J. Gandolfi, R. Nagle, and J.W. Clayton. 1981. Effects of inhaled chlorotrifluoroethylene and hexafluoropropene on the rat kidney. *Toxicol. Appl. Pharmacol.* 59:431-440.

Singer, L. and R.H. Ophaug. 1979. Concentrations of ionic, total, and bound fluoride in plasma. *Clin. Chem.* 25(4):523-525.

Weil, C.S. 1952. Tables for convenient calculation of median-effective dose (LD_{50} or ED_{50}) and instructions in their use. *Biom.* 8:249-263.

Zar, J.H. 1974. *Biostatistical Analysis.* Englewood Cliffs, NJ: Prentice Hall.

3.1.5 Protocol for the Acute Inhalation Toxicity Testing of Bel-Ray Syncom 1400

E. R. Kinkead

INTRODUCTION

Bel-Ray Syncom 1400 is a synthetic hydraulic fluid presently used in Naval operations. A highly water-soluble material, its major component is polyalkylene glycol. A minor component of Bel-Ray Syncom 1400, tert-octyl-*n*-phenyl-2-naphthylamine (Material Safety Data Sheet, Ciba-Geigy Corp.), has been reported to cause eye and skin irritation. In addition, the manufacturer of another minor component, alkylammonium mixed alkyl acid phosphate, has warned that prolonged contact to it may result in skin irritation (Material Safety Data Sheet, E.I. duPont deNemours & Co., Inc.).

Irritation and sensitization studies on Bel-Ray Syncom 1400 have been conducted by the THRU and reported by Gaworski and Horton (1985). Minor skin and eye irritation were reported at 4 and 24 h, respectively. However, signs of irritation had resolved by the next evaluation period. Bel-Ray Syncom 1400 produced no sensitization response in guinea pigs. Acute inhalation toxicity information is not available.

The Naval Air Engineering Center has begun operational testing with the Bel-Ray Syncom 1400 fluid. Personnel working in a compartment where the fluid was being used complained of headaches and dizziness. The fluid had been added to an open water tank at a concentration of 9% (by volume). Also present in the water was 0.2% of a 50/50 mixture of sodium nitrite and an emulsifier, sodium alkylsulfonylglycine. The air in the compartment was saturated with mist and steam, which contained the water-soluble Bel-Ray Syncom 1400.

The NMRI/TD requested that acute inhalation toxicity tests be performed on an aerosol of water containing 9% Bel-Ray Syncom 1400 and a 0.2% weight/volume mixture of sodium nitrite and sodium alkylsulfonylglycine. Male and female rats have been selected as the test animals for the inhalation tests, as recommended by the Environmental Protection Agency (U.S. EPA, 1982). Existing alternative methods to animal testing are inadequate for this study.

MATERIALS

Test Material

Bel-Ray Syncom 1400 and the 50/50 mixture of sodium alkylsulfonylglycine and sodium nitrite have been supplied by NMRI/TD. The components of Bel-Ray Syncom 1400 are listed in Table 3.1.5-1.

TABLE 3.1.5-1. COMPONENTS OF BEL-RAY SYNCOM 1400

Material	Approx. % Mass
Polyalkylene glycol	65.0
Ester (unidentified)	26.0
Sulfated caster oil	5.0
Carboxylic acid salt	2.5
Tert-octyl-*n*-phenyl-1-naphthylamine	0.5
Organic phosphoric acid ester	0.5
Alkyl ammonium mixed alkyl acid phosphate	0.3

Animals

Seven- to ten-week-old, male and female Fischer-344 rats will be used for this study. At the time of the exposures males will weigh between 150 and 300 g and females between 125 and 250 g. The approximately 240 rats needed for this study will be obtained from Charles River Breeding Labs, Kingston, NY. Upon receipt, the animals will be housed in Building 838 (Veterinary Sciences) for quarantine and quality control according to Air Force Standard Operating Procedures. The rats will be randomized according to a randomization regimen prepared by the NSI-ES Biometry Section. Toe clips will be used for identification.

The rats will be group housed by sex in plastic cages with wood chip bedding. They will be provided Purina Formulab 5008 feed and softened water (not to exceed one grain per gallon hardness, measured as calcium carbonate) *ad libitum*. The cage bedding will be changed twice per week.

This study will be monitored by a veterinarian. Any animal determined to be in pain or distress will be euthanized. No drugs will be administered, as they would interfere with the test results. At the end of the 14-day postexposure observation period, all animals will be euthanized by halothane inhalation.

METHODS

Acute inhalation toxicity testing will be performed on male and female Fischer-344 rats using a water emulsion containing 9% Bel-Ray Syncom 1400, 0.2% by weight of sodium nitrite and sodium alkylsulfonylglycine (Table 3.1.5-2). Respirable aerosols of the test material will be produced by compressed-air nebulization or ultrasonic fractionation methods. The method of choice will depend upon the chemical characterizations of the material. Aerosol concentration will be determined

gravimetrically. Aerosol particles will be collected on appropriate filters for volatile/nonvolatile proportion analysis, and particle size will be measured with cascade impactors.

Mortalities and visible signs of toxicity will be recorded. A 14-day LC_{50} with 95% confidence limits will be calculated using the probit analysis method of Finney (1971). Body weights will be recorded prior to exposure and on days 7, 10, and 14 postexposure. A repeated measures test will be used to compare body weights of exposed and control animals (Barcikowski, 1983).

All animals living at the termination of the observation period will be sacrificed. Gross pathological examinations will be performed on all rats exposed to the test agent. If deaths occur as a result of exposure, lung, trachea, liver and kidneys will be sampled for histopathological examination.

TABLE 3.1.5-2. EXPERIMENTAL DESIGN OF ACUTE INHALATION STUDY

Group	Exposure Time (h)	Concentration (mg/L)	No. of Animals	Assessment
I – Limit Test	4	5.0	5 male 5 female	Body weight, toxic signs, mortality
II – Control	4	Air only	5 male 5 female	Same as Group I
III – LC_{50}[a]	4	To be determined[b]	10 male 10 female	Same as Group I plus histopathology if mortality occurs
IV – Control[a]	4	Air only	10 male 10 female	Same as Group III

[a] Group III and IV studies will be performed only if deaths occur at the limit test.
[b] At least three exposure concentrations will be used and spaced appropriately to produce a range of toxic effects and mortality rates sufficient to permit the determination of an LC_{50}.

REFERENCES

Barcikowski, R.S. 1983. *Computer Packages and Research Design* Vol. 1, *BMDP.* Lanham, MD: University Press of America.

Finney, D.J. 1971. *Probit Analysis.* Cambridge, England: Cambridge University Press.

Gaworski, C.L. and J.R. Horton. 1985. Evaluation of the acute Irritation and sensitization potential of the hydraulic fluid, Bel-Ray Syncom 1400. University of California-THRU Letter Report, 12 September 1985.

U.S. Environmental Protection Agency. 1982. *Health Effects Test Guidelines.* Report No. EPA 560/6-82-001. Washington, DC: Office of Pesticides and Toxic Substances.

3.1.6 Evaluation of the Sensitization and Acute Skin Irritation Potential of Navy Watch Cap Samples

E. R. Kinkead and S. S. Henry

INTRODUCTION

During manufacture of Navy watch caps, dermal, eye, and pulmonary irritation were reported in factory workers. Before distributing of the finished caps to Navy personnel, the question of their potential for producing symptoms was of interest.

The objective of this study was to evaluate the dermal irritation and skin sensitization potentials for the watch caps in question, and to compare the findings with (1) those obtained for watch caps from previous lots in which there have been no reported adverse effects and (2) to standard solutions of proven irritants. The results obtained from this study will be evaluated to determine if the lot of watch caps in question has dermal irritation potential.

MATERIALS AND METHODS

Animals

Male, New Zealand White rabbits, weighing between 2 and 3 kg, were purchased from Clerco Research Farms, Cincinnati, OH. Male, albino, Hartley guinea pigs, weighing between 200 and 250 g upon receipt, were purchased from Murphy Breeding Laboratory, Inc., Plainfield, IN. Quality-control determinations, made during a two-week quarantine period, showed the animals to be in acceptable health. The animals were housed individually, the guinea pigs in plastic cages with wood-chip bedding, the rabbits in wire-bottom stainless steel cages. Water and feed (Purina Rabbit Chow #5320 and Purina Formulab #5025) were available *ad libitum*. Ambient temperatures were maintained at 21 to 25°C, and the light/dark cycle was set at 12-h intervals.

Test Materials

The watch cap samples were supplied by the NMRI/TD.

Preparation of Watch Cap Samples

The watch cap material was prepared for irritation and sensitization testing by cutting patches of approximately 9 cm^2 (0.5 gm) and 2 cm^2 (0.1 gm), respectively. The physical characteristics of watch cap sample #86-072-1 appeared to be different from the other two watch cap samples, #86-084-1 and #86-090-01. While preparing patches of watch cap sample #86-072-1, a considerable amount of lint was produced. Also noticed with this sample was extensive fraying at the cut edges, which was not seen with the other two watch cap samples.

Primary Skin Irritation

Three watch cap samples were tested using the standard EPA patch test on intact and abraded rabbit skin. Moderate (5% sodium lauryl sulfate), mild (30% acetic acid), and negative (distilled water) skin irritants were tested concurrently with the watch cap samples. Six rabbits per test material were clipped of hair on the dorsal trunk area 24 h prior to exposure to allow for the skin to recover from any abrasion that resulted from the clipping. Two areas on the back, one on each side, were designated as patch-test areas. The sites on the right side were abraded and those on the left remained intact. Adjacent areas of untreated skin served as controls.

The watch cap samples were moistened with 0.5 mL of distilled water. A 0.5-mL dose of the liquid materials or 0.5 g of watch cap sample was applied over each site. The treated areas were then covered with gauze patches, which were secured with strips of surgical tape. The entire area was covered with dental dam and secured with Elastoplast® tape. After 24 h, all wrappings were removed. Skin reactions were evaluated and scored following the method of Draize (1959).

Evaluations were made at 24, 48, and 72 h following application. The total score of the 3 observations for all rabbits was divided by 18 to yield a primary irritation rating. The primary irritation rating was interpreted using the NIOSH skin test rating (Campbell *et al.*, 1975).

Skin Sensitization

Two watch cap samples (#86-090-01 and #86-072-1) were tested using a modification of the Maguire method (Maguire, 1973) as described by Horton *et al.* (1981). A known sensitizing agent, chlorodinitrobenzene (CDNB) (Horton *et al.*, 1981), and a negative sensitizer, Carbowax 4000 (Carpenter *et al.*, 1971), were tested concurrently with the watch cap samples. Ten male, Hartley albino guinea pigs, six to eight weeks of age, were used for each test material.

An area on the back of each animal, directly above the forelegs, was shaved with electric clippers. The remaining fur was chemically removed with a chemical depilatory on the morning of the first exposure. Test materials (0.1 mL of liquids, 0.1 g watch cap samples dampened with 0.1 mL of distilled water) were applied to this area, and covered by gauze and dental dam. The patch was secured with adhesive tape. The first insult patch was removed after two days, and a second similar insult patch was applied. Two days later, this patch was removed, a total of 0.2 mL of Freund's adjuvant was injected intradermally into 2 to 3 sites adjacent to the insult area, and a new test patch was applied. Three days later the final test patch was applied; it was removed after two days. A two-week induction period followed the removal of the final application.

After the two-week induction period, the flanks of the animal were clipped and challenged with the test material on one side, and distilled water on the other. The challenge applications were

not occluded. The skin response at these sites was recorded at 24 and 48 h after application according to the evaluation method provided in Table 3.1.6-1. Any animal eliciting a score of 2 or more at the test solution challenge site at the 48-h scoring was rated a positive responder.

TABLE 3.1.6-1. GRADING OF SKIN REACTIONS IN THE MAGUIRE GUINEA PIG SENSITIZATION TEST

Erythema	Edema
0 - None	0 - None
1 - Very slight pink	1 - Very slight
2 - Slight pink	2 - Slight
3 - Moderate red	3 - Moderate
4 - Very Red	4 - Marked

RESULTS

Primary Skin Irritation

Groups of six rabbits each were used to measure the degree of primary dermal irritation of intact and abraded skin to three watch cap samples and positive and negative control compounds. The three watch cap samples did not produce a primary skin irritation response on either intact or abraded rabbit skin. Positive and negative control materials produced the expected responses.

Skin Sensitization

Groups of ten guinea pigs each were tested for sensitization response to two watch cap samples and positive and negative control compounds. The guinea pigs exposed to the control substances responded as expected. Nine of ten guinea pigs challenged with CDNB responded at 24 h, with 50% still showing a positive response at 48 h. Guinea pigs treated with Carbowax 4000 showed no response at both 24 and 48 h. Three guinea pigs exhibited a mild response to watch cap #86-090-01 at 24 h; however, no erythema was evident at 48 h. Two guinea pigs responded to watch cap sample #86-072-1 at 24 h. Only one animal showed a moderate response, which persisted throughout the 48-h exposure period.

To determine whether these watch cap reactions were a sensitization response, a rechallenge of three guinea pigs from the CDNB and watch cap groups was performed one week after the initial challenge. Two groups of three guinea pigs each from the Carbowax 4000 group were rechallenged with watch cap samples and to serve as naive controls. The CDNB-treated guinea pigs responded to the rechallenge of the compound in a manner similar to the first challenge. However, the watch cap

samples produced no sensitization response in any of the guinea pigs as a result of the rechallenge exposure.

DISCUSSION

Application of watch cap samples #86-072-1, #86-084-1, and #86-090-01 to the intact and abraded skin of rabbits produced no signs of erythema or edema at any of the evaluation periods. On the basis of these results neither watch cap material would be considered a primary skin irritant.

The skin sensitization test was designed to evaluate the potential of materials to act as antigens. Applications of small quantities of antigenic material over a period of time induces antibody production. The induction potential can then be evaluated by grading the response to a challenge administration of the material. The 48-h response of one guinea pig in the group exposed to watch cap sample #86-072-1 indicated that the material may be a weak sensitizer. In questionably sensitized guinea pigs, rechallenge is an effective confirmatory step in determining sensitization (Gad *et al.*, 1986). The negative response to the rechallenge with the watch cap samples indicated an absence of antibody production and, therefore, a lack of sensitization potential.

A physical difference in the fibers of sample #86-072-1 was noted during the cutting of test samples. The cutting and handling of the sample resulted in a fine lint production, which was not noted when preparing the other two watch cap samples. Therefore, it is possible that airborne particulate lint could be the cause of skin irritation or adverse effects that have been noted in workers handling the material.

REFERENCES

Campbell, K.I., E.L. George, L.L. Hale, and J.F. Stara. 1975. Dermal irritancy of metal compounds. *Arch. Environ. Health.* 30:168-170.

Carpenter, C.P., M.D. Woodside, E.R. Kinkead, J.M. King, and L.J. Sullivan. 1971. Response of dogs to repeated intravenous injections of propylene glycol 4000 with notes on excretion and sensitization. *Toxicol. Appl. Pharmacol.* 18:35-40.

Draize, J.H. 1959. Dermal Toxicity, In: The Staff of the Division of Pharmacology of the Federal Food and Drug Administration. *Appraisal of the Safety of Chemicals in Food, Drugs and Cosmetics*, pp. 46-49. Austin, TX: The Editorial Committee of the Associates of Food and Drug Officials of the United States.

Gad, S.C., B.J. Dunn, D.W. Dobbs, C. Reilly, and R.D. Walsh. 1986. Development and validation of an alternative dermal sensitization test: The mouse ear swelling test (MEST). *Toxicol. Appl. Pharmacol.* 84:93-114.

Horton, J.R., J.D. MacEwen, E.H. Vernot, and A. Hall. 1981. *Comparisons of Skin Sensitization Methods: Landsteiner, Maguire and Guinea Pig Maximization.* AFAMRL-TR-81-131 (ADA-110633). Wright-Patterson Air Force Base, OH: Air Force Aerospace Medical Research Laboratory.

Maguire, H.C. 1973. The bioassay of contact allergens in the guinea pig. *J. Soc. Cosmet. Chem.* 24:151-162.

3.1.7 Mathematical Model of Hemolysis, Shape Transformation, and Intramembrane Aggregates in Human Erythrocytes

C. D. Flemming

INTRODUCTION

Tri-n-butyl tin (TBT) has been shown to produce tin-containing aggregates and shape transformations in human erythrocytes (Porvaznik, *et al.*, 1986). The purpose of this study was to determine whether other organotin compounds exhibit similar characteristics. The organotin compounds studied were tripropyltin chloride (TPT), tetrabutyltin (TTBT), triethyltin bromide (TET), di-*n*-butyltin dichloride (DBT), triphenyltin chloride (TPhT), and tri-*n*-butyltin methoxide (TMT).

Two analyses were performed using the hemolysis and shape transformation data generated as a result of erythrocyte exposure to TBT and related compounds. The first analysis examined the hypothesis that hemolysis was relative to the dosage of test compound. The second analysis searched for relationships between hemolysis, shape transformation, intramembranous tin aggregation, dose, and the identity of the compound.

METHODS

Hemolysis/dose relationship

The percent hemolysis for each compound was analyzed by a repeated measures design using BMDP4V software (Dixon,1983). The between factor was dose (μM) and the within factor was time (h). Multiple comparisons of the data were conducted using a Scheffe technique (Barcikowski, 1983).

Doseleffect relationship

A log-linear model was used to examine the relationships between hemolysis, shape transformation, intramembranous tin aggregation, dose, and identity of the compound (Bishop,1975). This model is applicable to ordinal data such as the response data obtained in this study. The analysis began (Model 1) by assuming no associations. All pairwise associations were determined and their corresponding statistics (degrees of freedom and chi-squared) were subtracted from Model 1. The pairwise association that had the smallest of all significant probabilities ($p < 0.05$) became Model 2. Using Model 2 as the new base model, other pairwise associations were formed and the associated statistics (degrees of freedom and chi-square) were subtracted from Model 2. The model with the smallest significant probability was chosen as Model 3. Model building continued until there were no more significant probabilities. The above procedures were conducted using BMDP4F software (Dixon, 1983).

Because percent hemolysis is a continuous variable and the other variables were not continuous, hemolysis data were transformed using the following scheme. The hemolysis category at each dose was established by using the mean at 4 h. The mean differed significantly ($p < 0.05$) from the zero dose at 4 h for all but the first category. The four established categories for hemolysis were 1 – slight (<4% and $p < 0.05$), 2 – mild (<25% and $p < 0.05$), 3 – moderate (>25% and <75% and $p < 0.05$), and 4 – severe (>75% and $p < 0.05$).

RESULTS

Hemolysis vs. dose and time

Hemolysis data were statistically analyzed and values categorized as defined in the methods section. The results are shown in Table 3.1.7-1.

TABLE 3.1.7-1. HEMOLYSIS VERSUS DOSE AND TIME

Compound	Dose	Time (hours)	Probability	Type of Hemolysis
TBT	25 µM	0.5	0.0000	mild
		1	0.0000	moderate
		1.5	0.0000	severe
		2	0.0000	severe
		3	0.0000	severe
		4	0.0000	severe
	10 µM	1	0.0025	mild
		1.5	0.0000	mild
		2	0.0000	mild
		3	0.0000	moderate
		4	0.0000	moderate
	5 µM	3	0.0353	mild
		4	0.0059	mild
	150 µM	0.5	0.0006	mild
		1	0.0000	moderate
		1.5	0.0000	severe
		2	0.0000	severe
		3	0.0000	severe
		4	0.0000	severe
TPT	100 µM	1	0.0000	mild
		1.5	0.0000	moderate
		2	0.0000	moderate
		3	0.0000	severe
		4	0.0000	severe

(continued)

TABLE 3.1.7-1. (Continued)

Compound	Dose	Time (hours)	Probability	Type of Hemolysis
TPT	50 µM	1.5	0.0101	mild
		2	0.0000	mild
		3	0.0000	mild
		4	0.0000	moderate
TTBT	5,000 µM	0.5	0.0000	moderate
		1	0.0000	moderate
		1.5	0.0000	severe
		2	0.0000	severe
		3	0.0000	severe
		4	0.0000	severe
	1,000 µM	0.5	0.0000	moderate
		1	0.0000	moderate
		1.5	0.0000	moderate
		2	0.0000	moderate
		3	0.0000	severe
		4	0.0000	severe
	500 µM	0.5	0.0000	mild
		1	0.0000	moderate
		1.5	0.0000	moderate
		2	0.0000	moderate
		3	0.0000	moderate
		4	0.0000	moderate
TpHT	2,500 µM	0.5	0.0000	moderate
		1	0.0000	moderate
		2	0.0000	severe
		3	0.0000	severe
		4	0.0000	severe
	1,000 µM	0.5	0.0000	mild
		1	0.0000	mild
		1.5	0.0000	mild
		2	0.0000	moderate
		3	0.0000	moderate
		4	0.0000	moderate
	500 µM	0.5	0.0000	mild
		1	0.0000	mild
		1.5	0.0000	mild
		2	0.0000	mild

(continued)

TABLE 3.1.7.1. (Continued)

Compound	Dose	Time (hours)	Probability	Type of Hemolysis
TpHT	500 μM	3	0.0000	moderate
		4	0.0000	moderate
	100 μM	2	0.0371	mild
		3	0.0000	moderate
		4	0.0000	moderate
TET	5,000 μM	0.5	0.0000	severe
		1	0.0000	severe
		1.5	0.0000	severe
		2	0.0000	severe
		3	0.0000	severe
		4	0.0000	severe
	2,500 μM	0.5	0.0000	mild
		1	0.0000	moderate
		1.5	0.0000	moderate
		2	0.0000	moderate
		3	0.0000	severe
		4	0.0000	severe
	1,000 μM	1	P<0.01	mild
		1.5	P<0.01	mild
		2	P<0.01	mild
		3	P<0.01	mild
		4	P<0.01	mild
DBT	5,000 μM	0.5	0.0000	moderate
		1	0.0000	moderate
		1.5	0.0000	moderate
		2	0.0000	moderate
		3	0.0000	moderate
		4	0.0000	moderate
	1,000 μM	1	P<0.01	moderate
		1.5	P<0.01	moderate
		2	P<0.01	moderate
		3	P<0.01	moderate
		4	P<0.01	moderate
DBT	500 μM	1.5	P<0.01	moderate
		2	P<0.01	moderate
		3	P<0.01	moderate
		4	P<0.01	moderate
	100 μM	4	0.0111	moderate

Dose/effect relationships

Table 3.1.7-2 gives the results for the model studies using variables listed in the methods section. Because both Models 1 and 2 in Table 3.1.7-2 are not significant but their difference is, there is an implied relationship between hemolysis and tin aggregation consistent with microscopic observations. To look for further relationships, Model 3 examines the pairwise relationship between shape and hemolysis, with dose and identity of compound independent of each other. The difference between Models 2 and 3, the contribution of shape and hemolysis, is significant. This indicates an additional relationship between shape and hemolysis. The only variables found to be statistically associated in all organotin compounds at all doses were intramembranous tin aggregates with hemolysis ($p = 0.0048$) and shape transformation with hemolysis ($p = 0.0268$).

TABLE 3.1.7-2. FIVE-FACTOR ASSOCIATION MODEL IN ORGANOTIN-EXPOSED ERYTHROCYTES

Model Number	Model Proposed[a]	Degrees of Freedom	Chi-Square	Probability Statistic
1	d, c, a, s, h (no association among variables)	3665	583.67	1.0000
2	d, c, ah, s (pairwise associations)	3605	570.75	1.0000
1-2	difference between Models 1 & 2	60	12.92	0.0048
3	d, c, ah, sh (step two)	3647	543.51	1.0000
2-3	difference between Models 2&3	15	27.24	0.0268

[a] d = dose, c = compound, a = aggregates, s = shape, h = hemolysis, sh = association between shape and hemolysis, ah = association between aggregates and hemolysis.

DISCUSSION

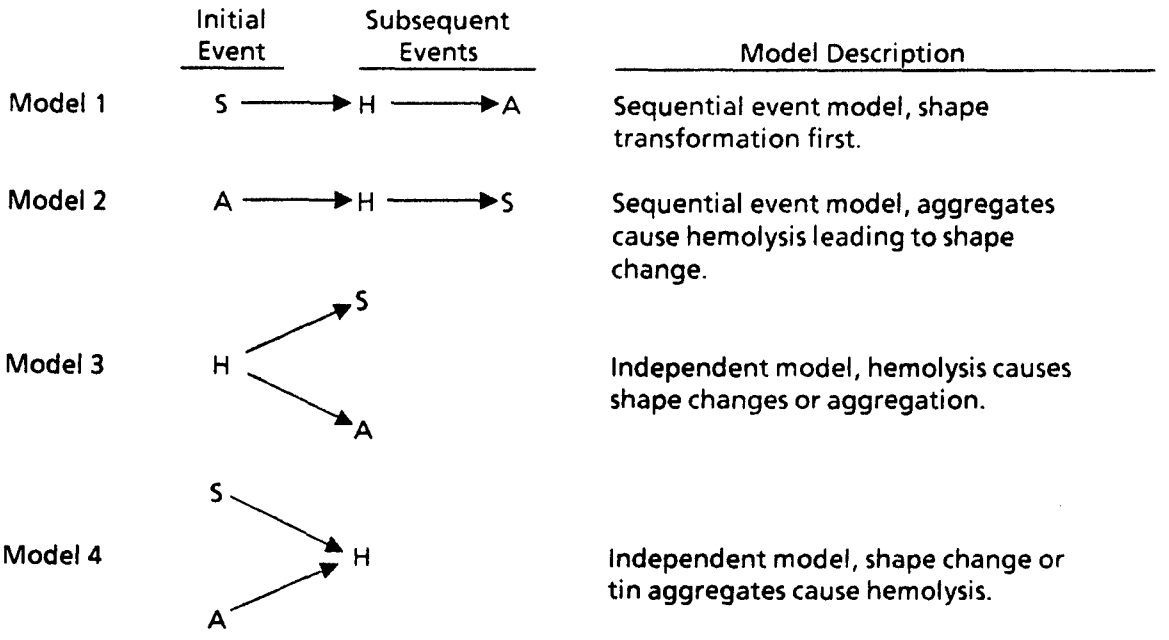
Organotin compounds examined in this study exhibited a relative order of potency for induction of ***in vitro*** hemolysis in human erythrocytes as follows:

TBT > TPT > TTBT > TPhT > TET > DBT > TMT.

The log-linear analysis indicated four causal models relating shape transformation (S), hemolysis (H), and tin-containing aggregates within membranes (A; Figure 3.1.7-1).

Model 4 fits the data and observations presented here. Shape transformation and organotin aggregation occur independently of each other and before hemolysis can proceed. Prior to this statistical modeling effort, Model 4 was the only mechanism suggested for hemolysis. However, the data suggest three other models with no obvious basis for excluding any of them. The results of applying the log-linear analysis indicate that Model 4, with independent shape transformation and tin aggregation as necessary precursors to hemolysis, is the valid model. This approach to data

analysis has allowed a nonquantitative observation to be confirmed analytically in such a manner that it can be used as a hypothesis for further experimentation. Light and electron microscopic observations of treated erythrocytes support the idea of independent processes of shape transformation and organotin aggregation leading to hemolysis. It is interesting that data analysis demonstrated no association between tin-containing aggregates and shape transformation or between tin-containing aggregates and concentration.



Legend:

S Shape Transformation of Red Blood Cells
H Hemolysis of Red Blood Cells
A Aggregation of Red Blood Cells
→ Effect of Event to the Left of Arrow

Figure 3.1.7-1. Possible Causal Models.

REFERENCES

Barcikowski, R.S. 1983. *Computer Packages and Research Design* Vol. 1., BMDP Lanham, MD: University Press of America.

Bishop, Y., S. Fienberg, and L.R. Forte. 1975. *Discrete Multivariate Analysis: Theory and Practice.* Cambridge, MA: MIT Press.

Dixon, W.J. 1983. *BMDP Statistical Software Manual.* Berkeley, CA: University of California Press.

Porvaznik, M., B.H. Gray, D. Mattie, A.G. Jackson, and R.E. Omlor. 1986. The ultrastructural localization of tri-n-butyltin in human erythrocyte membranes during shape transformation leading to hemolysis. *Laboratory Investigation* 54:254-267.

3.1.8 The Evaluation of Acute Skin Irritation of an Activated Peroxide Preparation Used For Decontamination

E. R. Kinkead, B. T. Culpepper, S. S. Henry

INTRODUCTION

The Navy expressed interest in a decontamination suspension for removal of chemicals from equipment and, possibly, from personnel. The material will be distributed in a dispenser containing the solid/peroxide clay ingredients and a liquid in a separate inner membrane, so that when squeezed, the slurry would mix, releasing the activated peroxide. An applicator top, much like that on liquid shoe polish, would be used to apply the suspension to clothing or small equipment.

Hydrogen peroxide, a caustic component of sodium carbonate peroxide, causes skin and eye irritation at high concentrations. Hydrogen peroxide is the most toxic component of the decontamination mixture. It is the purpose of this study to evaluate the skin irritation potential of the decontaminant mixture. The expected route of exposure of the mixture would be primarily as a dermal agent in a dry or hydrated form. Therefore, initial testing of the decontaminant mixture involved primary irritation test of the slurry, or cream, on adult rabbits. In the event this particular compound is used for decontamination of personnel, this study may be included in preclinical tests because Good Laboratory Practices were followed. The rabbit was chosen as the test animal to conform with the requirements of the EPA (U.S. EPA, 1982). Existing alternative methods to animal testing are inadequate for this study.

MATERIALS AND METHODS

Animals

Upon receipt, six New Zealand White female rabbits, weighing between 2 and 3 kg, were quality control tested and found to be in acceptable health. The rabbits were housed singly in stainless steel cages with wire-mesh floors. Water (softened, not to exceed 1 grain/gallon hardness measured as calcium carbonate) and food (Purina Rabbit Chow # 5320) were given *ad libitum*. All rabbits were sacrificed at the termination of the observation period, and sections of exposed and control skin were sampled for histopathologic examination.

Test Materials

The test materials were supplied by the NMRI/TD. The test materials received are as follows:

Sample	NMRI/TD No.
DCON mix (per bag): 26 g sodium percarbonate 39 g clay blend	86-241-2, a-j
Solvent mix (per bag): 0.1% Triton X-100 15.0% butyl carbitol 1.0% didecyl dimethyl ammonium Remainder water Total volume per bag = 200 mL	86-241-1, a-i

The sample was prepared by thoroughly mixing one bag of solvent mix with one bag DCON mix. This was prepared within 30 min of use in the irritation test, as specified by NMRI/TD.

Prior to testing, the pH of the cream was determined. If the pH had been less than 2.0 or greater than 11.5, testing for irritation would not have been done due to predictable corrosive properties.

Primary Skin Irritation Test

Six female rabbits were topically dosed with 0.5 mL of the test agent to a patch area. This area was covered by 2.5 cm^2 of surgical gauze, two single layers thick, and the gauze was held in place with strips of Elastoplast® tape. The entire area was covered with dental dam and secured with Vetrap® and Elastoplast® tape. The patch remained in place for 4 h, at which time all wrappings and excess test material were removed.

Examination for signs of skin irritation were made at 4, 24, 48, and 72 h following application. Irritative effects were scored according to the method of Draize (1959). The total score of the 4 observations for all rabbits was divided by 24 to yield a primary irritation rating. The primary irritation rating was interpreted using the NIOSH skin test rating of Campbell *et al.* (1975).

RESULTS

After primary dermal irritation of intact skin to the test material, none of the rabbits showed any signs of erythema, edema, or necrosis at the 4-h observation period; however, for the remaining three observation periods, all of the rabbits showed signs of erythema with some edematous test sites. A total score of 54 recorded for the six rabbits for four observation periods gave a primary irritation rating of 2.25, which indicates the compound is too irritating for human skin contact.

DISCUSSION

The application of this test material to the intact skin of rabbits produced signs of erythema and edema with a primary irritation rating of 2.25. On the basis of these results this test material should be considered a primary skin irritant and precautions taken to prevent human skin contact.

REFERENCES

Campbell, K.I., E.L. George, L.L. Hale, and J.F. Stara. 1975. Dermal irritancy of metal compounds. *Arch. Environ. Health.* 30:168-170.

Draize, J.H. 1959. Dermal Toxicity. In: The Staff of the Federal Drug Administration, eds. *Appraisal of the Safety of Chemicals in Food, Drugs, and Cosmetics*, pp. 46-49. Austin, TX: The Editorial Committee of the Associates of Food and Drug Officials of the United States.

U.S. Environmental Protection Agency. 1982. *Health Effects Test Guidelines.* Report No. EPA 560/6-82-001. Washington, DC: Office of Pesticides and Toxic Substances.

3.2 TOXICOKINETICS AND PHARMACODYNAMICS

3.2.1 Chloroform Cytotoxicity

R. B. Conolly

INTRODUCTION

Chemical contamination of groundwater is an important problem at several Air Force bases. Cancer has been induced in test animals by exposing them to large amounts of certain contaminant chemicals every day for their entire lifetime. Only a few of these cancer-causing contaminants change the genetic material in the cell; most cause tumors in a more indirect manner. For example, chemicals such as chloroform cause extensive damage to liver and kidney cells. Many scientists believe that repetitive toxic damage in these tissues may ultimately lead to tumor growth. To set exposure guidelines for cytotoxic, indirectly carcinogenic chemicals, the risk assessment process must include an understanding of the relationship between the toxicity of a chemical to a tissue and the increased numbers of tumors produced in that tissue. This project will produce a quantitative model of tissue damage as related to tumor formation, and will provide the rationale for conducting cancer risk assessments with chloroform and similar chemicals.

To date, we have developed a computer model of the relationship between repeated exposure to cytotoxicants and subsequent tumor development. This model is roughly isomorphic with actual mammalian physiological and biochemical systems and may explain why chemicals such as chloroform, which is cytotoxic but not genotoxic, test positively in carcinogenicity bioassays (National Cancer Institute, 1976; Roe *et al.*, 1979; Palmer *et al.*, 1979; Heywood *et al.*, 1979; Jorgensen *et al.*, 1985). The model describes the mathematical relationships between

- *in vivo* cytotoxicant exposure and tissue dose of parent compound and metabolites,
- tissue dose and cell death,
- cell death and regenerative hyperplasia, and
- regenerative hyperplasia and normally scheduled replication and the accumulation of genetic mutations.

The model described here is a combination of three discrete models describing pharmacokinetics, cytotoxicity, and carcinogenesis. These discrete models are: (1) the physiologically based pharmacokinetic (PB-PK) model of Ramsey and Andersen (1984), (2) the cytotoxicity model described in this manuscript, and (3) the two-stage carcinogenicity model of Moolgavkar and Knudson (1981).

The PB-PK model was used as a pharmacokinetic driver for the cytotoxicity model to provide accurate linkage between *in vivo* toxicant exposure and tissue doses of parent compounds and reactive metabolites. This PB-PK model has been validated for several volatile halogenated compounds and solvents (Ramsey and Andersen, 1984; Clewell *et al.*, 1985; Gargas *et al.*, 1986).

The cytotoxicity component of the model contains equations describing depletion of a generic macromolecule (MM) by a reactive metabolite of the parent compound. MM is subsequently resynthesized by a feedback-controlled enzymatic system. Depletion of MM leads to cell death, which is followed by regenerative hyperplasia. The description of MM depletion was based on a model of hepatic glutathione depletion and resynthesis that has been previously described (Andersen *et al.*, 1986). A model of cytotoxicity mediated by parent chemical bioactivation and macromolecule depletion, which was developed by Reitz (1987), also contributed to this work.

In Moolgavkar and Knudson's carcinogenicity model (Moolgavkar and Knudson, 1981) cellular replication incurs a risk of error, leading to mutations in daughter cells. We have used cytotoxicity, as described above, to force regenerative hyperplasia and thereby increase the rate of mutation accumulation. Simulated data are presented showing how cells containing one and two mutations accumulate as a function of time and toxicant exposure.

METHODS

Computer Hardware and Software

The mathematical model was written in Advanced Continuous Simulation Language (ACSL; Mitchell & Gauthier Associates, Inc., Concord, MA) and runs on an IBM-AT.

Modeling of Cytotoxicity

In Ramsey and Andersen's PB-PK model (Ramsey and Andersen, 1984), the liver was used as the target organ for cytotoxic effects (Figure 3.2.1-1). This choice of target organ is not important per se for the results and conclusions reported here, and was used only as a matter of convenience. Model parameters were scaled for a 250-g rat, and the liver was modeled as a population of 10^8 cells with basal death and birth rates of 10^{-3}/h. Liver cells contained a generic macromolecule (MM) essential for cell viability. (In a model validated for a specific cytotoxicant whose mechanism of action involved macromolecular binding, the generic MM would be replaced with a description of the actual macromolecule(s) involved. Validation experiments using chloroform as the model cytotoxicant are planned.) The rate of hepatic metabolism of parent compound was dependent on the rate of toxicant delivery to the liver and the Michaelis constants for metabolism. Depletion of MM was modeled by describing a second-order reaction of the parent compound metabolite with MM. Cell death was linked to MM depletion by a normal curve (Hastings, 1955) where >99.999% of the area under the curve lay between the maximum and minimum possible levels of a MM. The cumulative percentage area under the curve, corresponding to the degree of MM depletion, defined

the expected rate of cell death. The liver responded to unscheduled cell death, i.e., cell death induced by the toxicant, by increasing the rate of cell division until the steady-state level of 10^8 cells was regained.

Modeling of mutation accumulation in hepatocytes is described below. Hepatocytes with no mutations (N1 cells) and those with one mutation (N2 cells) were modeled as having the same basal death and birth rates, being equally susceptible to cytotoxicant, and responding to unscheduled cell death, i.e., cytotoxicity, in the same manner. Cells with two mutations (N3 cells) were considered insensitive to cytotoxicant.

The cytotoxicity model did not describe the maximum amount of cell death that could be tolerated without death of the animal. This could easily be done but was not considered necessary for this exercise.

Figures 3.2.1-1, 3.2.1-2, and 3.2.1-3 illustrate various aspects of the cytotoxicity model. Figure 3.2.1-4 is useful for studying model function.

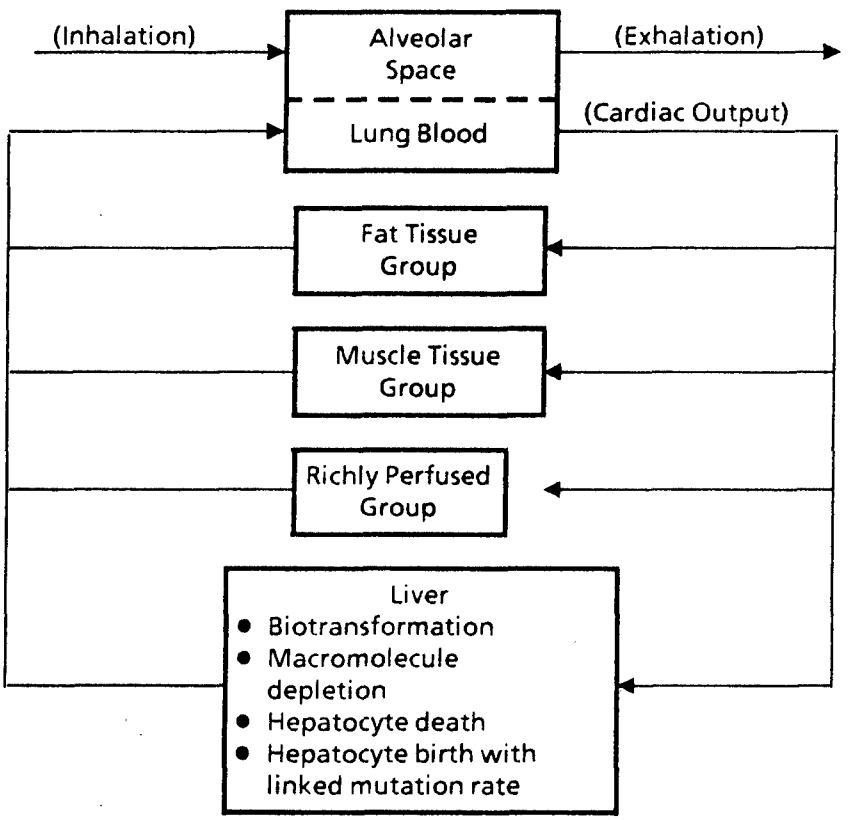


Figure 3.2.1-1. Schematic of the Biologically Based Pharmacokinetic Model of Ramsey and Andersen (1984) as Adapted to Describe Hepatic Cytoxicity and Mutation Accumulation. Each compartment is defined by biologically realistic parameters.

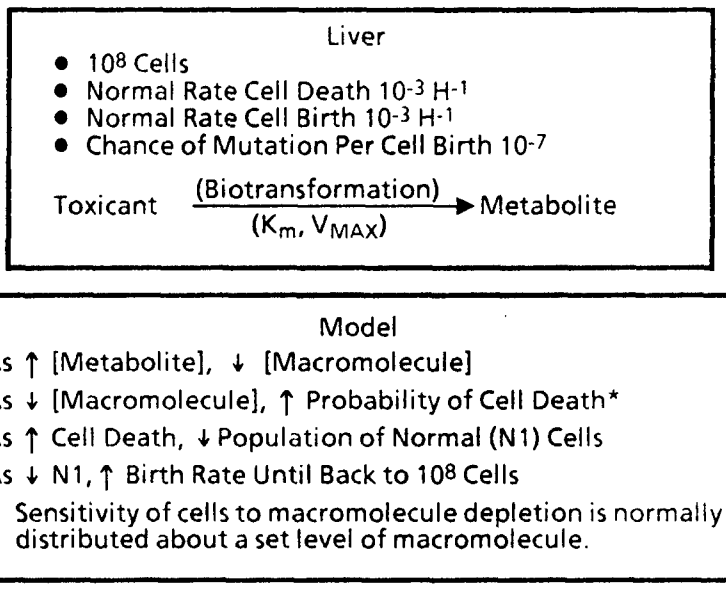


Figure 3.2.1-2. Detail for the Cytotoxicity Component of the Model. The choice of liver was one of convenience. This description of cytotoxicity could be applied equally well to other tissues.

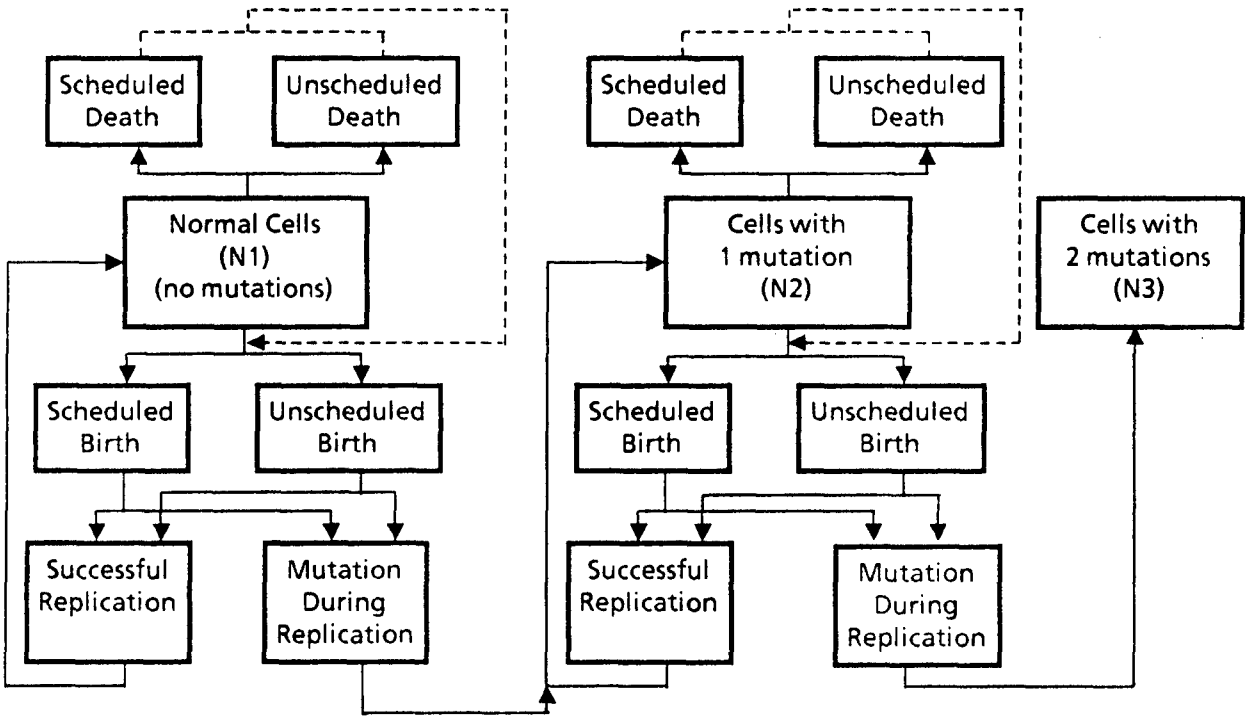


Figure 3.2.1-3. Adaptation of the Moolgavkar and Knudson Two-Stage Model for Carcinogenicity (Moolgavkar and Knudson, 1981) to the Pharmacokinetic/Cytotoxicity Model. N1 and N2 cells have the same basal birth and death rates, and N3 cells are immortal. "Unscheduled" events are those due to cytotoxicity.

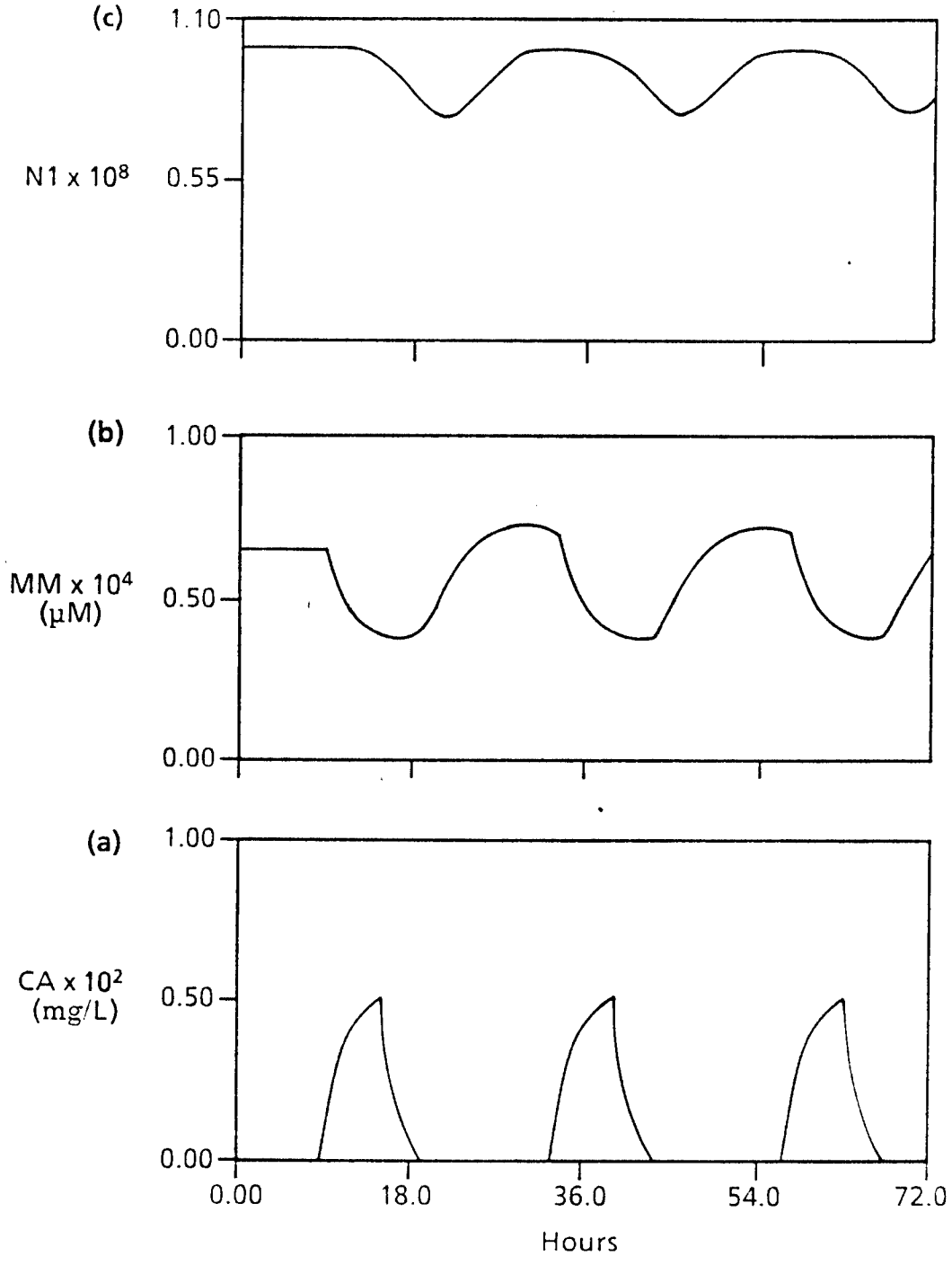


Figure 3.2.1-4. Temporal Relationships of *In Vivo* Toxicant Exposure, Macromolecule Depletion, and Number of Normal (N1) Cells. (a) Daily 6-h exposure to parent toxicant is tracked by the spikes in its arterial concentration (CA). (b) Depletion of macromolecule (MM) by a metabolite of the parent and resynthesis of MM as CA declines. (c) N1 death as MM is depleted, and consequent regenerative hyperplasia.

Modeling the Relationship of Cell Birth to Mutation Accumulation

A premise of the model is that regenerative hyperplasia consequent to cytotoxicity increases the rate at which mutations accumulate in the target organ, and that some of these mutations support tumor development. This premise is based on the assumption that a small fraction of cellular replications go wrong, resulting in mutant progeny. In the model, normal hepatocytes (N1 cells) have a 10^{-7} chance of suffering a mutation during replication, and cells containing one mutation (N2 cells) replicate with the same kinetics as N1 cells and as also have a 10^{-7} chance of suffering a mutation during replication. Cells having two mutations (N3 cells) are assumed to be tumorigenic without further mutation. These relationships are illustrated in Figure 3.2.1-3.

Generic Nature of Cytotoxicity Model

We know of no body of data suitable for use in mathematical modeling of the relationships between reactive metabolite production, depletion of critical cellular macromolecules, rates of cell death and regeneration, and accumulation of mutations. Therefore, we defined a "generic macromolecule," MM, and estimated reasonable quantitative relationships between the amount of parent compound metabolized, depletion of MM, cell death and birth, and mutation accumulation. Because the definition of MM is not linked to a biochemical mechanism of toxicity for a particular chemical, the model's descriptions of cytotoxicity and mutation accumulation are also generic. For these reasons, even though the PB-PK model is well validated for a number of compounds, we present the simulations of cytotoxicity and mutation accumulation in terms of exposure to a model cytotoxicant rather than to any particular chemical.

RESULTS

Figure 3.2.1-4 illustrates the qualitative relationships between cytotoxicant exposure, depletion of MM, cell death, and regenerative hyperplasia. Simulation of repeated, 6 h/day inhalation exposure to parent compound results in corresponding spikes in its arterial concentration (CA). As CA increases, MM concentration decreases. This decrease in MM is due to its destruction by the cytotoxic metabolite of the parent compound. As the concentration of cytotoxic metabolite decreases, MM is resynthesized and its concentration increases. The model simulates an overshoot or rebound effect where the level of MM rises above its pre-exposure concentration. This type of rebound effect is seen after toxicant-induced depletion of hepatic glutathione (GSH; Wong, K.-L. and Klaassen, 1981), which we have modeled for related projects. Although not shown in Figure 3.2.1-4, the MM concentration would return to the pre-exposure basal level between 24 and 48 h after a single 6-h exposure. The decreasing concentration of MM is followed by a corresponding decline in N1, the number of normal hepatocytes. N1 returns to its basal level, approximately 10^8

cells, after MM is resynthesized. In all, Figure 3.2.1-4 illustrates several cycles of toxicant-driven cell death and regenerative hyperplasia.

Figure 3.2.1-5 simulates the accumulation of cells with one mutation (N2 cells) over time in toxicant-exposed and control groups. Note the linear nature of N2 accumulation with time and the demonstration that toxicant exposure can greatly increase the rate of N2 accumulation.

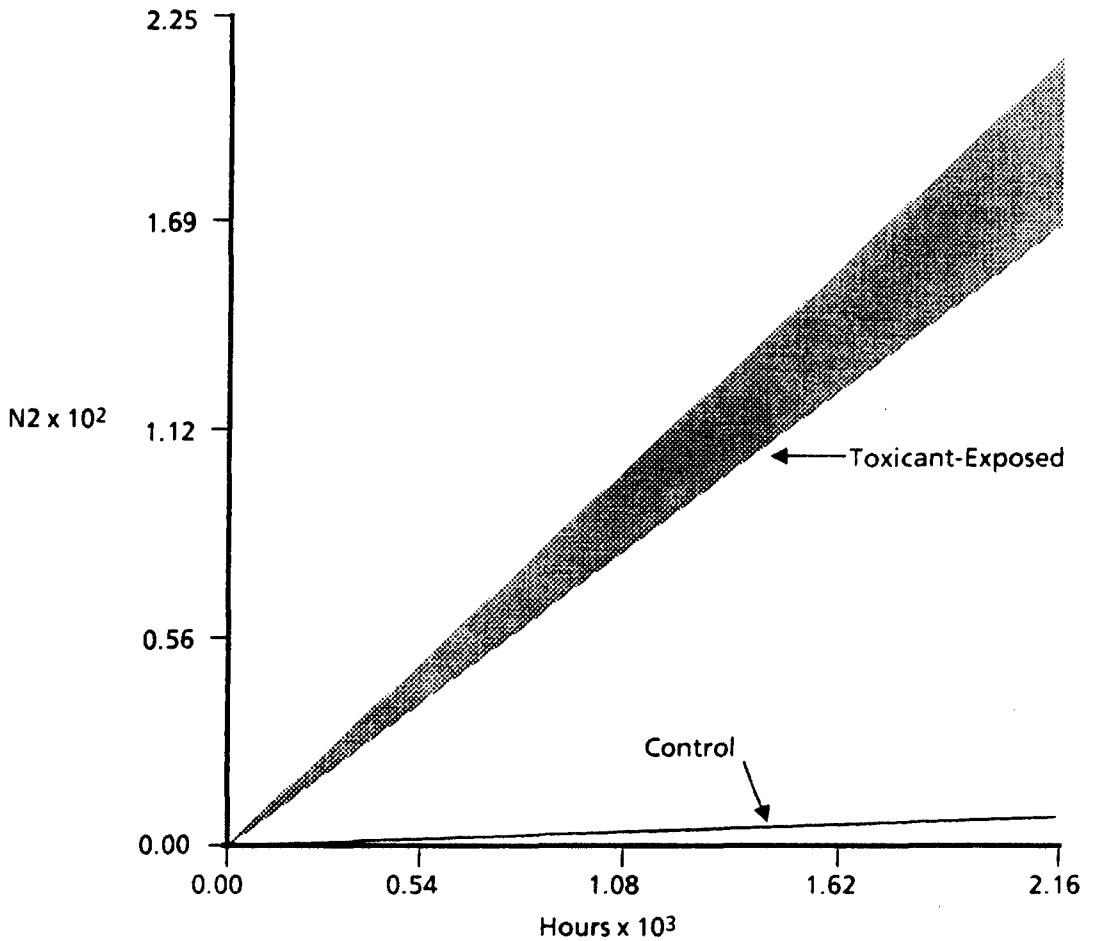


Figure 3.2.1-5. Simulated 90-day Accumulation of Cells with One Mutation (N2) in Control and Toxicant-Exposed Groups. The daily fluctuation in the number of N2 cells in the toxicant-exposed group reflects the cycles of cell death and regenerative hyperplasia caused by each 6-h exposure to toxicant.

Simulated rates of accumulation of cells with two mutations (N3 cells) are shown in Figure 3.2.1-6. Notice that Figure 3.2.1-6 uses log-log scaling, and that the lines depicting N3 accumulation with time are described by a power function.

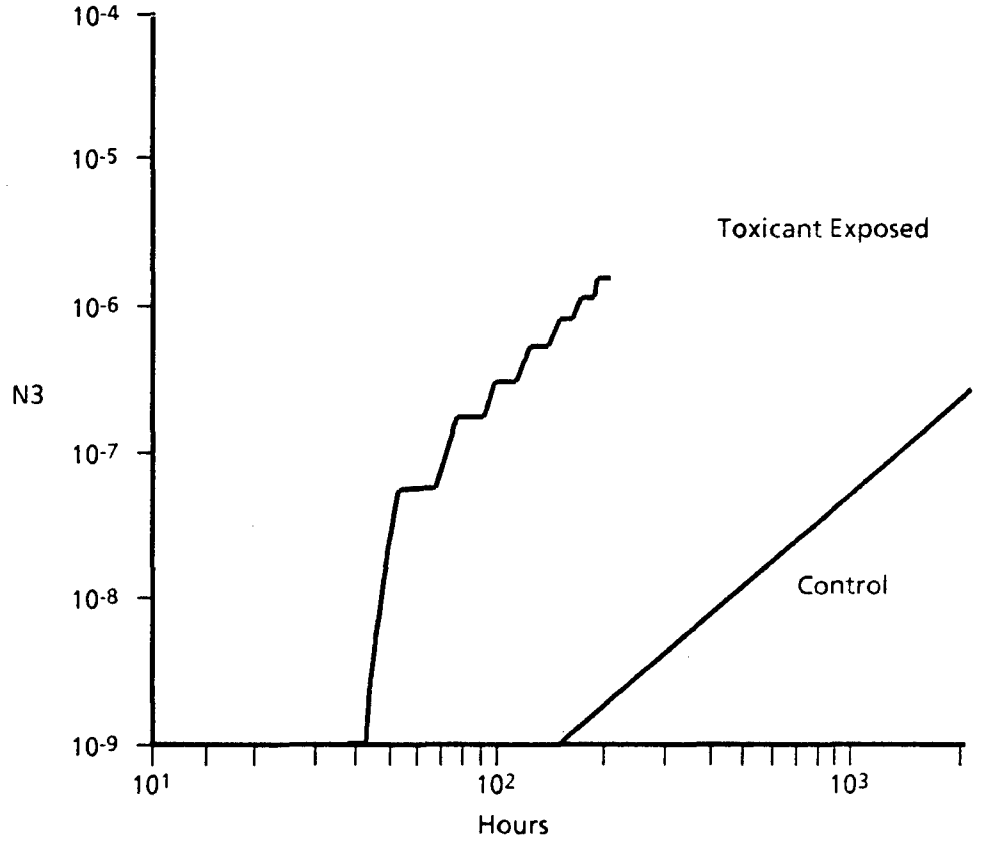


Figure 3.2.1-6. Simulated 90-day Accumulation of Cells with Two Mutations (N3) in Control and Toxicant-Exposed Groups. The toxicant-exposed group suffered about 20% cell death/day, which was completely replaced within 24 h by regenerative hyperplasia. This regenerative hyperplasia leads to the difference between the control and toxicant-exposed groups. In both the control and exposed groups, N3 accumulation is proportional to the square of time.

Figure 3.2.1-7, which uses arithmetic scaling, shows the numbers of N1, N2, and N3 cells occurring during the simulation of 1 year of 6 h/day toxicant exposures.

The model uses a cumulative probability function to obtain the rates of birth of N2 and N3 cells. This means that fractional N2 and N3 cells are produced during a simulation. For example, in Figure 3.2.1-6 it can be seen that about 10^{-4} cells were produced during 90 days simulated exposure, causing 20% cell death/day. This number, 10^{-4}, actually represents the average expected number of tumors per rat. The poisson distribution can be used to determine the numbers of rats having 0, 1, or more than 1 tumor.

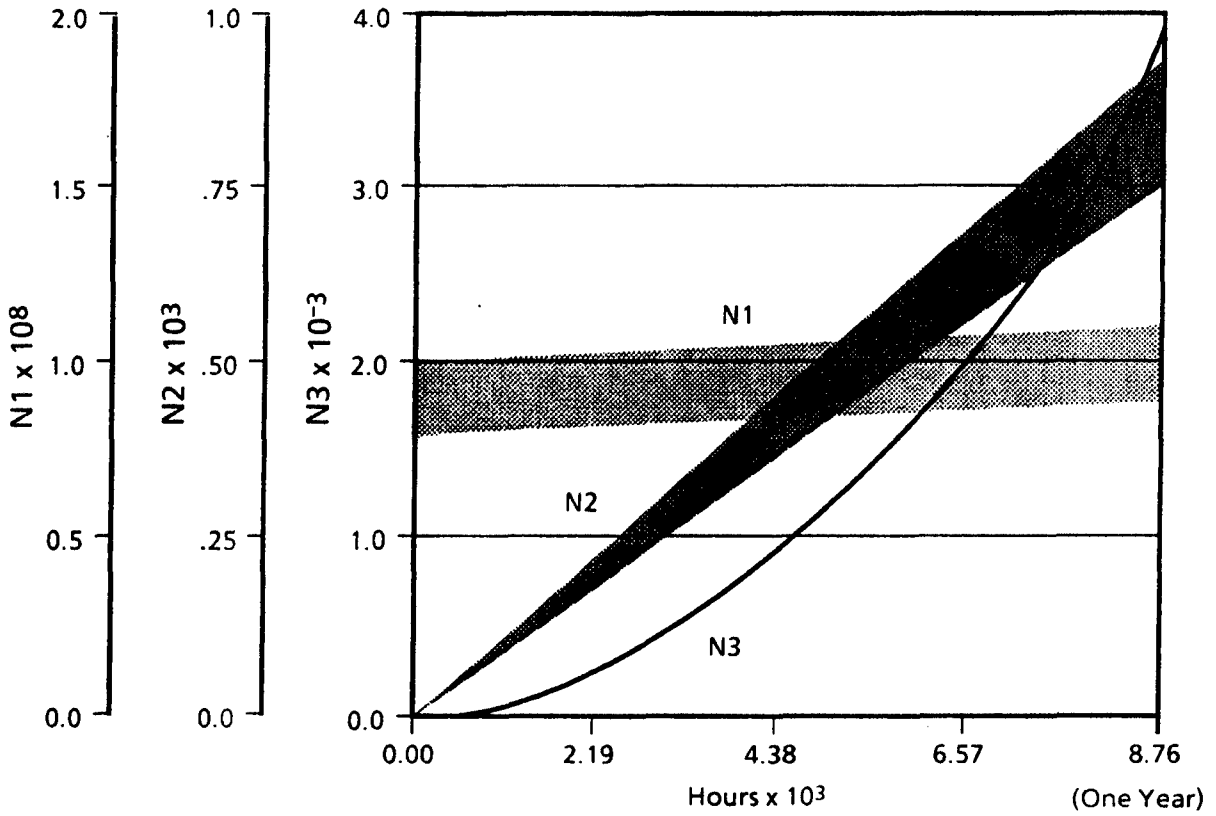


Figure 3.2.1-7. Simulation of 1 Year of Daily 6-h Inhalation Exposures to a Model Cytotoxicant. Daily fluctuations in N1 and N2 are due to cytotoxicity. Note that the rate of accumulation of cells with one mutation (N2) is linear with time, while accumulation of cells with two mutations (N3) depends on the square of time.

DISCUSSION

The model described here incorporates some straightforward assumptions about relationships between the tissue dose of toxicant, the depletion of a critical cellular macromolecule, the likelihood of cell death, and the probability of mutations occurring during regenerative hyperplasia. These assumptions reflect well-characterized phenomena. Depletion of hepatic GSH is known to be linked to increased probability of cell death (Docks and Krishna, 1976; Wells *et al.*, 1980; Mitchell *et al.*, 1973). It is generally accepted that mutation does occur as a consequence of mistakes during cellular replication (Tsutsui *et al.*, 1978; Tsutsui *et al.*, 1981; Crawford *et al.*, 1983).

The model suggests that repeated episodes of cytotoxicity increase the rate at which mutations accumulate in a target tissue. Moreover, cells that contain two mutations and would presumably be more likely to be tumorigenic (Moolgavkar and Knudson, 1981) accumulate as a function on the square of time, while the accumulation of cells with one mutation is linear.

A significant advantage of this model is that the tissue dose of cytotoxic metabolite is related to *in vivo* parent compound exposure by the well-validated PB-PK model (Ramsey and Andersen, 1984; Clewell *et al.*, 1985; Gargas *et al.*, 1986). Modeling of cytotoxicity and other target-organ consequences of *in vivo* exposure has little meaning if tissue dose of the ultimate toxicant is not well characterized.

Limitations of the Model – What Is Modeled and What Isn't

Several processes that would affect cytotoxicity and mutation accumulation have not been specifically described in this model. For example, the model was exercised repetitively in order to simulate chronic exposures of up to one year (Figures 3.2.1- 5,6,7). For these long-term simulations it may be necessary to model the aging process in the rat. Changes in the size of the fat compartment and in the capacity for parent compound metabolism with age would affect pharmacodynamic behavior. The effect of DNA repair capacity on the mutation rate could also be considered. The possibility that N2 cells have growth advantages, i.e., altered death and birth rates and different sensitivity to MM depletion relative to N1 cells, may give rise to N2 birth rates different from those simulated here. Immune surveillance of phenotypically altered cells probably affects the N2 and N3 populations, and modeling of this parameter may be necessary for a comprehensive description. The model does not estimate the biological significance of the simulated mutations. Because not all mutations would be expected to predispose the cell to clonal growth, the accumulation of N3 cells is probably more rapid than the accumulation of tumorigenic cells. These factors may all be important for accurate simulation of rates of cytotoxicity and mutation accumulation. However, the relative shapes of the curves describing the change in N1, N2 and N3 populations with time (Figure 3.2.1-7) should not differ between a model specifically describing these factors and the one described here.

Model Validation

As noted above, the PB-PK model of Ramsey and Andersen (1984), which is used as a pharmacokinetic driver for the cytotoxicity model, has been well validated. We have begun the process of obtaining actual data on the quantitative relationships between $CHCl_3$ biotransformation, cell death, cell regeneration, and the consequent accumulation of mutations. These data will be used to develop a $CHCl_3$ cytotoxicity model, which will provide a strong test of the hypothesis that $CHCl_3$ cytotoxicity is responsible in whole or in part for the positive outcomes seen in $CHCl_3$ carcinogenicity bioassays (National Cancer Institute, 1976; Roe *et al.*, 1979; Palmer *et al.*, 1979; Heywood *et al.*, 1979; Jorgensen *et al,* 1985). At the present stage of its development, the generic model (see Methods) can be used to examine aspects of the quantitative relationships between depletion of critical cellular macromolecules, cytotoxicity, cell death and replication, and mutation accumulation.

It should be noted that the model does not describe the accumulation of genetic mutations caused by direct interactions of parent compound or metabolites with DNA. However, the model does provide a framework that could readily be modified to describe these factors. In addition to genetic mutation models, one could develop pharmacodynamic models of epigenetic carcinogenesis, i.e., cancer causation where the primary toxic effect does not involve DNA.

In conclusion, this presentation illustrates the potential utility of using computer-based models of biological systems to study the relationships between cytotoxicity, cell replication, and accumulation of genetic damage. A particularly interesting result generated by the model described here is the dependence of the birth rate of cells containing two mutations on the square of time.

REFERENCES

Andersen, M.E., H.J. Clewell III, M.L. Gargas, and R.B. Conolly. 1986. A physiological pharmacokinetic model for hepatic glutathione (GSH) depletion by inhaled halogenated hydrocarbons. *The Toxicologist* 6:148, Abstract No. 598.

Clewell III, H.J. and M.E. Andersen. 1985. Risk assessment extrapolations and physiological modeling. *Toxicol. Ind. Hlth.* 1:111-131.

Crawford, B.D., J.C. Barrett, and P.O.P. Ts'o. 1983. Neoplastic conversion of preneoplastic Syrian hamster cells: Rate estimation by fluctuation analysis. *Molec. Cellular Biol.* 39:31-945.

Docks, E.L. and G. Krishna. 1976. The role of glutathione in chloroform-induced hepatotoxicity. *Exp. Molec. Pathol.* 24:13-22.

Gargas, M.L., H.J. Clewell III and M.E. Andersen. 1986. Metabolism of inhaled dichloromethanes *in vivo*: Differentiation of kinetic constants for two independent pathways. *Toxicol. Appl. Pharmacol.* 82:211-223.

Hastings, C. 1955. In: J.T. Hayward and J.P. Wong, Jr., eds. *Approximations for Digital Computers*, p. 26. Princeton, NJ: Princeton University Press.

Heywood, R., R.J. Sortwood, P.R.B. Noel, A.E. Street, D.E. Prentice, F.J.C. Roe, P.F. Wadsworth, A.N. Worden, and N.J. Van Abbe. 1979. Safety evaluation of toothpaste containing chloroform. III. Long term studies in beagle dogs. *J. Environ. Pathol. Toxicol.* 2:835-851.

Jorgensen, T.A., E.F. Meierhenry, C.J. Rushbrook, R.J. Bull, and M. Robinson. 1985. Carcinogenicity of chloroform in drinking water to male Osborne Mendel rats and female B6C3F1 mice. *Fund. Appl. Toxicol.* 5:760-769.

Mitchell, J.R., D.J. Jollow, W.Z. Potter, J.R. Gillette, and B.B. Brodie. 1973. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J. Pharmacol. Exp. Ther.* 187:211-217.

Moolgavkar, S.H. and A.G. Knudson, Jr. 1981. Mutation and cancer: A model for human carcinogenesis. *J.N.C.I.* 66:1037-1052.

National Cancer Institute. 1976. Carcinogenesis bioassay of chloroform. NTIS No. PB246018/AS. Bethesda, MD: National Cancer Institute.

Palmer, A.K., A.E. Street, F.J.C. Roe, A.N. Worden, and N.J. Van Abbe. 1979. Safety evaluation of toothpaste containing chloroform. II. Long term studies in rats. *J. Environ. Pathol. Toxicol.* 2:821-833.

Ramsey, J.C. and M.E. Andersen. 1984. A physiologically-based description of the inhalation pharmacokinetics of styrene in rats and humans. *Toxicol. Appl. Pharmacol.* 73:159-175.

Reitz, R.H. 1987. The role of cytotoxicity in the carcinogenic process. In: B.E. Butterworth and T.J. Slaga, eds. *Nongenotoxic Mechanisms in Carcinogenesis*, Banbury Report 25. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Roe, F.J.C., A.K. Palmer, A.N. Worden, and N.J. Van Abbe. 1979. Safety evaluation of toothpaste containing chloroform. I. Long term studies in mice. *J. Environ. Pathol. Toxicol.* 2:799-819.

Wells, P.G., R.C. Boerth, J.A. Oates, and R.D. Harbison. 1980. Toxicologic enhancement by a combination of drugs which deplete hepatic glutathione: Acetaminophen and doxorubicin (adriamycin). *Toxicol. Appl. Pharmacol.* 54:197-209.

Tsutsui, T., J.C. Barrett, and P.O.P. Ts'o. 1978. Induction of 6-thioguanine- and ouabain-resistant mutations in synchronized Syrian hamster cell cultures during different periods of the S phase. *Mutat. Res.* 52:255-264.

Tsutsui, T., B.D. Crawford, and P.O.P. Ts'o. 1981. Comparison between mutagenesis in normal and transformed Syrian hamster fibroblasts. Difference in the temporal order of HPRT gene replication. *Mutat. Res.* 80:357-371.

Wong, K.-L. and C.D. Klaassen. 1981. Relationship between liver and kidney levels of glutathione and metallothionein in rats. *Toxicol.* 19:39-47.

3.2.2 Setting Up and Evaluating the Efficacy of an Isolated Ventilated Perfused Rat Lung Preparation

A. Vinegar and D. M. Brown

INTRODUCTION

The nonrespiratory metabolic functions of the lung have received increased attention in recent years. Knowledge of these functions and interest in the fate of xenobiotic substances have resulted in increased use of the isolated ventilated perfused lung (IVPL) preparation as an attempt to localize the contribution of the lungs to the metabolism of these substances. In some cases, the lung itself may be the target for toxic effects of the parent compound and/or its metabolites.

Because of the influence of other organs, there is no adequate method of studying pulmonary metabolic activity *in vivo*. *In vivo* sampling of pulmonary arterial and venous concentrations of chemicals is of questionable value (Rochester *et al.*, 1973; Tierney, 1974). The IVPL preparation maintains relatively normal anatomical and physiological relationships between the lung and its circulation, allowing an investigator to study the effects of agents administered into either the lungs' circulation or the inhaled air supply. Recent reviews of the use of the IVPL preparation, with discussion of methods used and compounds studied, are found in Mehendale *et al.* (1981) and Niemeier (1984).

Results obtained from metabolic studies using the IVPL will serve as input data to pharmacokinetic physiological models. The compartments of these models are discrete organ systems with associated blood flows, volumes, and partition coefficients (Andersen, 1981). To use data from the IVPL as input to these models the quantitative and qualitative results must be consistent with actual *in vivo* conditions. In fact, as the models improve, the modeling process itself may indicate when the IVPL results are not consistent with *in vivo* metabolism. The necessity for accurate physiological data requires that the IVPL apparatus be well described physiologically and biochemically and that every effort be made to optimize those variables in the preparation that most influence normal metabolism. The degree of variability in expected results is epitomized by the multitude of ways the IVPL has been set up and used in different laboratories (Mehendale *et al.*, 1981; Niemeier, 1984).

We describe herein the procedures for setting up, maintaining, and monitoring an IVPL and the criteria that will be used for making further changes in the preparation.

MATERIALS AND METHODS

General Procedure

The basic approach to setting up the IVPL preparation will follow that described by Dalbey and Bingham (1978) for the rat lung. Their procedure is a modification of that described for the rabbit lung by Niemeier and Bingham (1972).

Male, Fischer-344 rats will be anesthetized with ketamine/xylazine. Blood will be collected from the vena cava just caudal to the heart, and will be pooled from several animals to make up a volume of 25 mL. The number of blood-donor animals, including the lung donor, will vary from four to five animals per preparation. The trachea, pulmonary artery, and left auricle of the lung-donor animals will be cannulated, and the lung will be removed and cleared of extraneous tissues. The lung will be placed into a water-jacketed glass "thorax" with the tracheal and pulmonary arterial cannulas vented externally. The arterial connection is made to a blood column, topped by a water-jacketed blood reservoir. The vertical distance of the surface of the blood in the reservoir to the hilum of the lung represents the driving pressure for the blood through the lung. The temperature of the water in the thorax and blood reservoir jackets is maintained at 37°C. The blood passing through the lung drips out the left auricular cannula into the bottom of the thorax. The level of blood in the reservoir is sensed by a level detector, and is maintained by activating a tubing pump that returns blood to the blood reservoir from the bottom of the thorax. A constant infusion of glucose into the blood reservoir compensates for glucose utilization. Likewise, pH is maintained by infusing bicarbonate at a constant rate. To fine-tune the pH, a pH meter controls the delivery of CO_2 to the air stream from which the lung breathes. The lung is ventilated by maintaining a negative pressure in the thorax, to maintain a functional residual volume, and by creating further negative swings in pressure with a rodent ventilator connected in reverse.

Blood/Perfusate Monitoring

The blood circulating through the IVPL will be monitored for a number of parameters. These parameters will be selected based on their relevance as indicators of biochemical or cellular mechanisms of the blood or the lung, or of interactions between the two. Not all parameters will be monitored simultaneously because of the limited volume of blood that can be removed from the preparation. The frequency of monitoring of a given parameter may change as its relevance to the IVPL is defined. The selection of some of the parameters is based on IVPL literature (Niemeier and Bingham, 1972). Comparisons with published data will be useful for assessing differences between methodologies. The perfusion circuit of the apparatus will be operated first with heparinized plasma, then with heparinized whole blood, then with heparinized whole blood perfusing an unventilated, and finally, a ventilated lung. Certain laboratory analyses that will be used are

applicable only to specific preparations (i.e., it is senseless to run a hematocrit on plasma). The analyses to be done appear in the following table. Analyses are divided into categories, and the significance of each category is briefly summarized in Table 3.2.2-1.

TABLE 3.2.2-1. PARAMETERS TO BE MONITORED IN BLOOD CIRCULATING THROUGH THE ISOLATED VENTILATED PERFUSED RAT LUNG PREPARATION

Category	Analysis	Significance
Formed elements of blood	Cell count (RBC, WBC, Platelet) Hemoglobin Hematocrit Hemolysis	Measures of cell destruction (hemolysis), platelet adhesion to glass, total gas transport capability of the perfusate, intravascular coagulation.
Blood gases	pH pO_2 pCO_2 O_2 saturation Base excess CO_2 (meg/L)	Monitors of membrane diffusion (lung and blood gas exchange), metabolic acidosis may be computed utilizing Sigaard-Anderson relationship
Serum electrolytes	Sodium Potassium Chloride Calcium Magnesium Phosphorus	Important in maintaining perfusate osmolarity, pH, cell membrane mechanics, vasomotor tone, and trace-metal reactions
Blood proteins	Albumin Total protein	Important in the maintenance of perfusate osmolarity. Denaturation of plasma proteins in extyracorpeal devices is theorized to produce pulmonary edema
Coagulation monitor	Heparin	Monitor of adequate anticoagulation of perfusate
Blood lipids	Cholesterol	Important indicator for some drug metabolites
Metabolic requirements and products	Blood urea nitrogen Creatinine Glucose Lactic acid	Indicators of normal metabolism
Bilirubin	Total bilirubin	Pigment release from RBCs is one indicator of hemolysis

Lung Monitoring

The condition of the lung will be assessed during the course of each experiment by monitoring certain physiological parameters. Transpulmonary pressure will be monitored by continuously measuring the pressure change between the trachea and the inside of the artificial thorax. Flow will be measured using a pneumotachograph placed in series with the tracheal cannula. The electrical flow signal will be integrated to obtain tidal volume. Dynamic compliance and pulmonary resistance will be calculated from the tidal tracings of volume, flow, and pressure. The compliance determinations will indicate changes in lung stiffness, and the resistance determinations will indicate changes in large airway diameter.

Evaluation of Efficacy of IVPL

One of the most common problems affecting the life of an IVPL is pulmonary edema. Some investigators have stopped collecting data at an early time to keep from reaching some subjective endpoint such as change in appearance of the lung. Other investigators have used diminution of blood flow as a criterion for stopping the preparation. By measuring certain initial parameters, we hope to be able to pick several objective criteria for determining when the IVPL is no longer viable and, furthermore, to lengthen the usable life of the preparation.

Animal Data

Fischer-344 rats, weighing between 150 and 250 g will be purchased from the Charles River Breeding Labs, Wilmington, MA.

Pathologic Evaluation

Lungs from ventilated perfusions will be examined by light and electron microscopy to evaluate changes that may result from the total IVPL procedure. Lungs will be prepared for light and electron microscopy by SOPs for THRU and the Ultrastructural Research Laboratory of Pathology Services (AAMRL/THT).

Study Plan

In order to identify any problems with the preparation, this study will be done in four phases. The first will involve running pooled plasma through the apparatus and the second phase will be with pooled whole blood. During the third phase we will use lungs and perfuse them with whole blood but not ventilate them, and in the fourth phase we will examine perfused, ventilated lungs. This stepwise process will allow us to determine where changes in the preparation are likely to take place. A final report will be submitted with our recommendations for use of the IVPL preparation in studies involving the metabolism of selected xenobiotic compounds.

RESULTS

These studies have just begun but the following preliminary results and observations have been obtained. Concern over hemolysis caused by the type of tubing pump usually used for IVPL preparations led to the design by THRU and fabrication of a variably occlusive pump. Initial indications were that total cell destruction was less than 3% after 4 h of pumping.

Studies of the thermodynamics of the original reservoir-thorax system demonstrated that thermal stability was inadequate, due in part to the large mass of stainless steel of which the pump head was made. A heating tape was placed around the pump head, and automatic control was applied to the pump controlling water jacket temperature. Blood temperatures can now be maintained within 1°C.

Early runs of the reservoir-thorax apparatus showed that blood evaporation in the open venous return system would pose a continuing problem. Dissection of rats indicated that cannulation of the pulmonary veins for pulmonary venous return is more feasible than previously expected. The reservoir and thorax were reconfigured to allow active venous return, the first step toward preparing a "closed" circulatory system. A closed system will be required for studying volatile chemicals.

Preliminary blood and plasma studies indicated that the method by which donor blood is acquired is critical to the preservation of normal blood components and chemistry. Specifically, arterial blood acquired from halothane anesthetized, heparinized male F-344 rats by substantial negative suction through a narrow bore needle is considerably hemolyzed, rendering interpretation of clinical values impossible. Platelet aggregation is also a problem with this technique. An arterial cannulation technique of slow hemorrhage of a well-hydrated, anesthetized, minimally heparinized animal is under study. Initial results by this method indicate an absence of hemolysis and reduced platelet aggregation.

A revised system for maintaining humidity of the lung exterior has been designed. The system is similar to that used in storage systems for human organ transplant.

Design of a Teflon/glass electromagnetic flow probe has been completed. Initial contact with a potential supplier indicated substantial interest in constructing such probes. A conventional electromagnetic flowmeter has been obtained, enabling direct measurement of blood flow *in vitro* and *in vivo* for various organ systems.

A new thorax assembly has been designed which incorporates stock glassware components, eliminating the need for specialized glass fabrication. The components are interchangeable to allow

perfusion of different organs and different small mammal species without compromise of basic design requirements for rodent lungs.

REFERENCES

Andersen, M.E. 1981. Pharmacokinetics of inhaled gases and vapors. *Neurobehav. Toxicol. Teratol.* 3:383-389.

Dalbey, W. and E. Bingham. 1978. Metabolism of trichloroethylene by the isolated perfused lung. *Toxicol. Appl. Pharmacol.* 43:367-277.

Mehendale, H.M., L.S. Angevine, and Y. Ohmiya. 1981. The isolated perfused lung – A critical evaluation. *Toxicology* 21:1-36.

Niemeier, R.W. 1984. The isolated perfused lung. *Environ. Health Persp.* 56:3335-41.

Niemeier, R.W. and E. Bingham. 1972. An isolated perfused lung preparation for metabolic studies. *Life Sci.* 2:807-820.

Rochester, D.F., W.A. Wichein, H.W. Fritts, P.R. Caldwell, M.L. Lewis, C. Gruntini, and J. G. Garfield. 1973. Arteriovenous differences of lactate and pyruvate across healthy and diseased human lungs. *Am. Rev. Respir. Dis.* 107:442-448.

Tierney, D.F. 1974. Lung metabolism and biochemistry. *Ann. Rev. Physiol.* 36:209-231.

3.3 TOXICOLOGY OF FUELS AND PROPELLANTS

3.3.1 Chemical Analysis for Dermal Hydrazine Exposures

D. L. Pollard and J. M. Adams

INTRODUCTION

A group of six rats was dermally exposed to hydrazine. This four-hour exposure was the most recent in a series of exposures designed to study the dermal absorption of hydrazine vapors. The chemical analysis for this exposure is reported here.

MATERIALS AND METHODS

Test Material

Hydrazine was supplied by American Scientific Products in a 500-g bottle, cat. # EKC902. The manufacturer was Eastman Kodak Company, Rochester, NY. Lot number C11E was used.

The quality control analysis was done on a Varian 3700 gas chromatograph, thermal conductivity detector, 10-ft x 1/8-in. stainless steel column packed with 60/80 mesh Tenax GC®. The column temperature was 85°C and the helium flow was 25 mL/min. The injection and detector temperatures were 110°C and 140°C, respectively. The detector current was 150 milliamps. The peak areas and percentages were determined by a Hewlett-Packard 3388A computing integrator.

Hydrazine is reactive and decomposes in the presence of light and air. For this reason, it was stored in brown bottles under nitrogen headspace. In spite of taking these precautions, however, a slight amount of decomposition could occur, so the test material was analyzed within a week before usage.

Generator

The generator consisted of a chamber air supply metered with a rotameter that had been calibrated with a chain compensated gasometer and a wet test meter. The contaminant was supplied through a tee in the air line using a 10-mL syringe driven with a syringe pump. Liquid delivery was measured from the syringe readings.

Analysis

Samples for determining the concentration of hydrazine in the chamber were collected through 1/8-in. polyflow tubing into a scrubber tower. The hydrazine was scrubbed with an absorber solution containing excess iodine, for complete reaction, and 40 g/L KI, 20 g/L Na_2HPO_4, and 6 g/L KH_2PO_4. The reacted absorber solution was pumped through the sample side of a Technicon

AutoAnalyzer II colorimeter and compared to the unreacted absorber solution pumped through the reference side. The colorimeter wavelength used to quantitate the hydrazine reaction products was 480 nm. The air sample and solutions were pumped with a Technicon Autoanalyzer II proportioning pump. The colorimeter output signal was recorded on a strip chart recorder.

The analysis calibration was checked within a week prior to use. The calibration was performed with Mylar® standard bags. During the exposure, 48 concentration averages, each of 5-min duration, were taken. The average hydrazine concentration was 231 ± 40 ppm, and the exposure system required 20 min to reach equilibrium.

Industrial Hygiene

Hydrazine in the air was sampled by an MDA analyzer Model 7080 located just outside the exposure hood. The air sample was pumped through the MDA analyzer supplied with hydrazine analysis tapes. These chemically impregnated tapes are sensitive to hydrazine. The flow was checked hourly with a flowmeter. The MDA response was recorded on a strip chart recorder. The MDA analyzer was calibrated and set to alarm at a level of 0.05 ppm hydrazine. The analyzer was run and records kept during the exposure.

CONCLUSION

Hydrazine vapor analysis was used for a series of dermal exposures. The method has demonstrated a standard deviation 4% of the mean at the 200-ppm reading. The overall generation/analysis system typically shows a 9% relative standard deviation after the chamber stabilizes.

3.3.2 Method Development for Sensitive Determination of Monomethylhydrazine in Biological Tissue

H. F. Leahy

INTRODUCTION

The current method under study for the analysis of monomethylhydrazine in biological fluids is a modification of a method developed for the simultaneous determination of hydrazine (H), monomethylhydrazine (MMH), and 1,1-dimethylhydrazine (UDMH) in air by derivatization/gas chromatography (Holtzclaw *et al.*, 1984). It was introduced following unsuccessful attempts to adapt the complex method presently in use for the analysis of hydrazine in biological fluids. This method is to be used for analyzing low concentrations of MMH in conjunction with a series of pharmacokinetic studies.

MATERIALS AND METHODS

Electron Capture Detector System (Halogenation Method)

The initial direction of this work was to use and modify as needed the method for the determination of hydrazine presently used by the Toxic Hazards Assessment (THA) group. This method is based on the reaction of *p*-chlorobenzaldehyde with hydrazine. When MMH is substituted for the hydrazine the product becomes *p*-chlorobenzaldehyde methylhydrazone (Hershey *et al.*, 1979). Following separation on a gas chromatograph (GC), an electron capture detector (ECD) was used rather than a flame ionization detector (FID) to increase the sensitivity of detection. Changes in solvents were dictated by use of the ECD. Further modifications of the basic method were introduced to purify the sample before injection on the GC. This complex multistep method included mixing, incubation with heat, adsorption on and elution from a clean-up column, evaporation, and, finally, solution in a measured volume of ethyl acetate.

Chemicals

The following chemicals are used with the halogenation method: monomethylhydrazine (MMH), *p*-chlorobenzaldehyde (*p*-CBA), and chromatographic-quality solvents, (benzene, ethanol, ethyl acetate, and glacial and acetic acid).

Instruments

The following instruments are used in the halogenation method: a Varian 3700 GC with a 30-m, wide bore, Supelcowax-10 capillary column (Supelco Inc.), an electron capture detector, and a Hewlett-Packard 3388A integrator.

Reaction Procedure

1. Mix the desired quantity of MMH or sample with solvent containing 3% (or less) *p*-CBA.

2. Heat at 50°C for 2.5 h.

3. Cool the sample or standard to room temperature, and rinse the reaction mixture onto the ChemElut column.

4. Elute the column with benzene, evaporate the benzene, dissolve the remaining material in ethyl acetate, and inject 1 µL into the ECD-equipped GC.

5. If the standard is for method study only, skip steps 3 and 4, then after cooling, dilute it to the desired concentration in ethyl acetate and inject 1µL into the ECD-equipped GC.

6. Use the integrated peak area for concentration determination.

Thermionic Specific Detector System (Hydrazone Method)

This method depends on the stoichiometric reaction of hydrazine with acetone to form 2-propanone methylhydrazone. The latter compound can be detected by a thermionic specific detector (TSD), following separation on a GC. The specificity of the detector for carbon-nitrogen bonds permits use of acetone as both solvent and reactant. The high concentration of acetone drives the reaction equilibrium to the right, causing the reaction to be essentially complete and independent of the MMH concentration.

Sample preparation for this method is minimal. A known mass or volume of tissue is added quantitatively to the reagent. The material is thoroughly mixed or homogenized, then centrifuged. The supernatant containing the hydrazone is decanted and analyzed by GC-TSD. Calibration standards are prepared in reagent and diluted to appropriate working ranges.

Chemicals

The following chemicals are used in the hydrazone method: monomethylhydrazine, acetone, and 2,6-di-*tert*-butyl-4-methylphenol (BHT).

Instruments

The following instruments are used in the hydrazone method: a Varian 3700 GC with Supelco 30-m wide-bore capillary columns (SPB 1 - non polar, SPB 20 - intermediate polarity, Supelcowax 10 - polar, and KOH-treated Supelcowax 10); a thermionic specific detector (TSD); and a Hewlett-Packard 3388A integrator.

Reaction Procedure

1. Mix the desired quantity of MMH or sample with acetone solution containing 5% distilled deionized water and 5.5 x 10^{-5} mL BHT.
2. Allow 5 min of reaction time.
3. Centrifuge the mixture to clarify the acetone layer.
4. Separate the supernatant from the precipitate after centrifugation.
5. Inject 1 µL of the supernatant into the TSD-equipped GC.
6. Calculate concentration from the integrated peak area .

RESULTS

The first series of experiments performed to study the reaction of MMH with *p*-chlorobenzaldehyde bypassed the cleanup steps and used relatively concentrated reactants, similar to the recommended method of standard preparation (Hershey *et al.*, 1979). The resultant derivative was then diluted to 10-100 ppm. Although a peak was identified by retention time and relative area, inconsistencies were the rule rather than the exception, and the results deteriorated as the concentration range of the MMH derivative was lowered. Derivatives were not found consistently when low concentrations of MMH (< 10 ppm) were reacted.

An alternate method, dependent on the use of a TSD, appeared to have the potential for approaching the requested sensitivity of a 10-ng/mL sample concentration. This procedure called for a simple reaction solution of acetone/water/BHT, which when mixed with MMH forms a derivitive containing the carbon-nitrogen bond necessary for GC/TSD analysis. When following the method described, the formation of the derivative posed no problem. The research has been directed at finding a satisfactory sample preparation technique as well as a chromatographic procedure. The chromatographic procedure should provide a good separation from interfering peaks as well as reproducibility of integrated area, especially when approaching the lowest concentration of interest.

Four column coatings were investigated. The nonpolar SPB-1 column gave rapid and good separation at higher concentrations, but at low concentrations the presence of an unresolved peak in some blood samples limited the sensitivity of the system. The low-intermediate polarity column SPB-20 also gave rapid separation but with peak tailing, thereby limiting the sensitivity. The polar Supelcowax 10 column gave a little slower separation but some undesirable peak tailing at the lowest concentrations. The final choice of the KOH-treated Supelcowax 10 was based on the need to

reduce the peak tailing associated with amine-type compounds observed on all the columns tested (Figure 3.3.2-1).

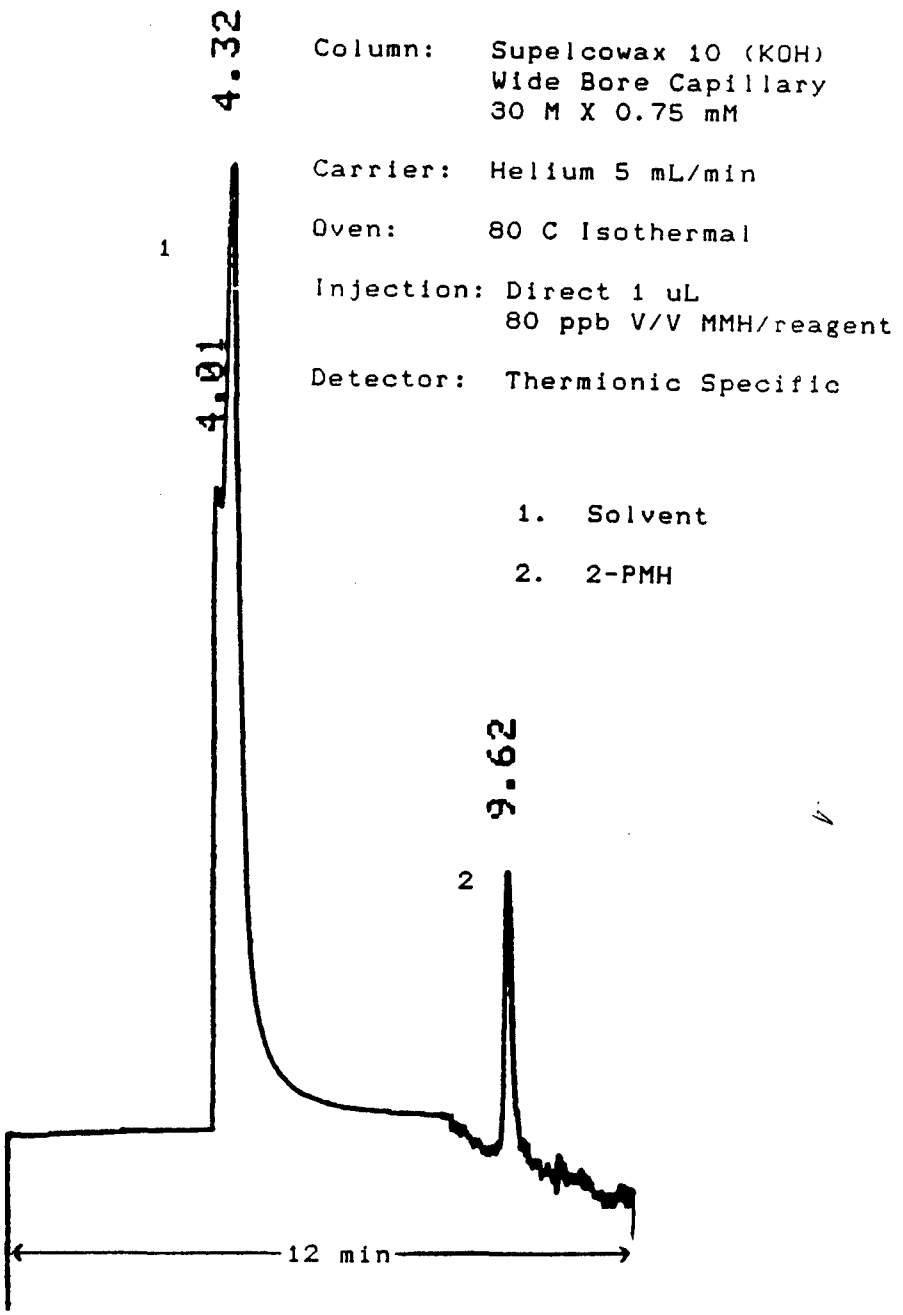


Figure 3.3.2-1. Chromatogram of 2-Propanone Methylhydrazone following the Method of Holtzclaw *et al.* (1984). MMH concentration of the reaction mixture was 80 ppb V/V.

Originally, the TSD system was reported to have a linear response for at least three orders of magnitude and a limit of sensitivity of 0.1 nmoles/mL when using a 3-μL injection volume (Holtzclaw *et al.*, 1984). Range-finding tests have given similar results in this laboratory when calibration standards were prepared by serial dilution of MMH in the reagent. The minimal detectable mass was found to be between 5×10^{-12} and 10×10^{-12} g. For precision of dilution as well as peak area interpretation the calibration concentrations were divided into a low (1-16 ppb V/V), medium (10-160 ppb V/V), and high (100-1600 ppb V/V) set of curves. One ppb V/V is equal to 0.85 ng/mL. To bring the MMH derivative (2-propanone methylhydrazone) concentration within the minimal detectable limit, the injection volume was 3 μL for the low-range standards and 1 μL for the medium and high ranges. The low-concentration samples were diluted 1 part to 5 with reagent, and the medium and high concentration samples were diluted 1 part to 10 with reagent.

While looking for critical steps in the chromatographic procedure a test was set up to check the effect of injection port penetration depth. Repeated injections of 1 μL of a 100-ppb standard (V/V) were made. The peak area using the shallow penetration, which did not reach the glass sleeve above the column, was 10% less than that for the other three depths. The standard deviation for the peak areas of the 15 other injections (5 at each depth) was 7.3% of the mean value; the standard error of the mean was 1.9% of the mean value.

A combined extraction and stability test was performed *in vitro*, spiking blood samples from control rats with saline-diluted MMH at 1-ppm V/V. Initially, dilutions of MMH in deaerated normal saline were tested. After addition of deaerated MMH to the reaction reagent in the same manner as for a sample, a similar response was found to that of dilutions in reagent alone. The final results of the blood extract, considering the extract from normal saline as 100%, were as follows: 89% after 30 s, 64% after 6 min, and 49% after 40 min. Neither EDTA nor heparin, when used as the anticoagulant, caused interference.

Three *in vivo* tests were performed before the final column was acquired, and were used to test the total system rather than to give definitive concentration determinations. Anesthetized rats were dosed by intraperitoneal injections of MMH solutions. Serial blood samples (0.1 mL) were drawn by heart puncture, mixed with reagent as rapidly as possible, then centrifuged. The supernatant was separated, then injected into the GC. The results of the mid-range test are presented in Figure 3.3.2-2.

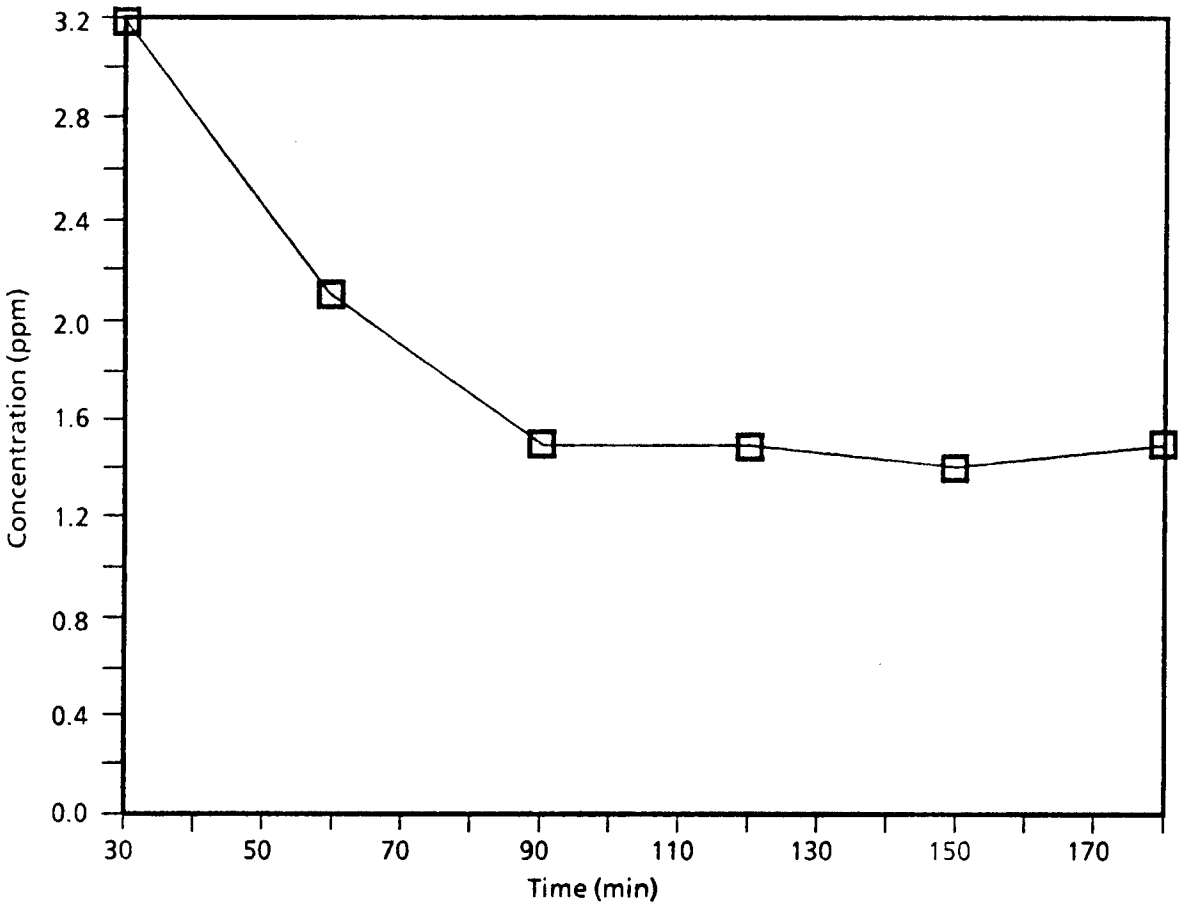


Figure 3.3.2-2. MMH Concentration in Blood following I.P. Injection of Rat with 8.5 mg/kg MMH.

DISCUSSION

The modification of the hydrazine method using the ECD failed to supply a consistent means of analyzing for MMH at the desired sensitivity. In the formation of the hydrazine derivative of *p*-chlorobenzaldehyde, the formation of a dihydrazone provides a more stable compound and twice the halogen for detection as is present for the MMH derivative. The use of acetic acid in the reaction mixture of the ECD method may have also contributed significantly to the negative results. Acetic acid is also presented initially in the TSD reference paper as a catalyst for formation of the hydrazine derivative. In the discussion section, however, recommendation is made that acetic acid be omitted when analyzing for MMH because acetic acid is thought to contribute to the instability of the MMH derivative. The oxygen concentration of the solution may also be critical, but was not controlled.

Initially, acetic acid was included in the acetone reagent as called for in the method, but its use was discontinued. The experience in this laboratory confirmed the conclusions of the original authors. Standards have been observed to be stable for more than a week when not including the acid. The water, which aids in trapping the MMH during air sampling, is not essential for the analysis

in the biological system. However, water was used so that both standards and biological samples would contain water. On one of the columns, excess water appeared to cause a double peak from the hydrazone. The addition of 2,6-di-*tert*-butyl-4-methylphenol (BHT), a mild reducing agent, contributes to the stability of the MMH derivative.

After deciding on the reaction procedure and detector type, we needed to choose a column for the GC that would result in reliable separation and good peak shape for sensitive and reproducible integration. The wide-bore capillary-type column was chosen because it permits a larger injection volume than normal capillary columns while giving better peak separation than packed-type columns. The three available columns were tested, beginning with the one already in the chromatograph. After determining that the 30-m wide-bore Supelco-Wax 10 column gave the best results, we obtained the KOH-treated version of that column. A functional sample reaction procedure for a wide range of sample concentrations was developed using either a five- or ten-fold dilution of blood by reagent and an injection volume of 1 or 3 μL of extract.

The highest successful injection volume under the present column conditions was 3 μL. Reproducible integration was lost as the baseline was compromised by a high slope from the excess solvent peak. Further sensitivity could probably be obtained by including a sample concentration step before injection.

The combined stability/extraction range-finding test demonstrated that the loss of MMH in the blood was a function of time rather than a significant deficiency in extraction. Previous work has demonstrated that the rate of MMH oxidation *in vitro* is species dependent (Leahy, 1970). Rats have been shown to have the slowest rate of the species tested. Further work may be needed before considering the results valid for other species.

Although the response for the MMH derivative in the range tested using the TSD system is linear, there is a potential for change in response. Because the sensitivity of the detector is a function of the hydrogen-to-air ratio and the applied bead current, the response to a particular concentration can change with a drift in either parameter. Increase of the hydrogen ratio will eventually cause the TSD-type detector to operate as an FID, thus losing the specificity of detection. Increase in bead current increases the detector sensitivity, but this occurs at the expense of the life of the detector. Normal operation is a compromise, and the limit is really determined by signal-to-noise ratio and reproducibility of the integrated signal. Calibration will demonstrate the type of slope and range to expect, but because of the potential for shift in response, a number of standards bracketing the sample concentrations will be required during a set of analytical runs to validate the slope. The conditions presently in use have a limit of sensitivity at 5×10^{-12} to 10×10^{-12} g per injection.

REFERENCES

Hershey, M.K., P.L. Sherman, and J.V. Pustinger. 1979. ***Development of Analytical Techniques for Toxicokinetic Research.*** AMRL-TR-79-89 (AD A-077729). Wright-Patterson Air Force Base, OH: Aerospace Medical Research Laboratory.

Holtzclaw, J.W., S.L. Rose, J.R. Wyatt, D.P. Roundbehler, and D.H. Fine. 1984. Simultaneous determination of hydrazine, methylhydrazine, and 1,1-dimethylhydrazine in air by derivatization/gas chromatography. ***Anal. Chem.*** 56:2952-2956.

Leahy, H.F. 1970. ***Monomethylhydrazine Effect on Blood, In Vitro.*** Proceedings of the 1st Annual Conference on Environmental Toxicology. AMRL-TR-70-102 (AD A-727022). Wright-Patterson Air Force Base, OH: Aerospace Medical Research Laboratory.

3.3.3 The Evaluation of the Teratogenic Potential of Otto Fuel II (OF II)

E.C. Kimmel

INTRODUCTION

Otto Fuel II (OF II) was introduced for use as a torpedo propellant in 1966, and is regarded as safe for this purpose in that it is relatively nonexplosive, insensitive to shock, and stable at temperatures up to 150°F. However, numerous adverse effects have been reported in humans (Stewart *et al.*,1974) and laboratory animals (Jones *et al.*, 1972) exposed to OF II or its principal constituent, propylene glycol dinitrate (PGDN; 1,2-propanediol dinitrate, approximately 75% by volume). Effects of acute exposure to OF II, PGDN, and similar nitrated esters include nasal congestion, eye irritation, skin discoloration, disorientation, vertigo, vasodilation, hypertension or narrowing of pulse pressure due to elevated diastolic pressure, dyspnea, and methemoglobinemia. These symptoms may occur as a result of either dermal contact, inhalation of OF II vapor or, most often, a combination of both modes of exposure (U.S.N. NAVMED P-5112, 1976).

There is a paucity of information regarding the health effects of chronic exposure to OF II. However, one investigation has shown hematologic toxicity in dogs, rats, and mice as a result of exposure to OF II vapor for approximately one year (Gaworski *et al.*, 1985). Given this information and what is known of the acute toxicity of OF II, there is ample justification for and interest in continued investigation of the toxicity of OF II.

One potential adverse health effect of OF II may be teratogenesis resulting from persistent OF II-induced methemoglobinemia and corollary hematologic dyscrasias effecting maternal oxygen transport (Wyman *et al.*, 1985) in pregnant females chronically exposed to OF II vapor. Consequently, a study was designed to investigate this possibility. The results of this study in conjunction with previous assessments of OF II toxicity should provide Naval personnel information pertinent to safe handling of this important propellant.

EXPERIMENTAL APPROACH AND DESIGN

Following the recommendations by the EPA Health Effects Test Guidelines (1982) for teratogenesis (developmental toxicity) studies, two mammalian species were selected for experimentation. These include the F-344 rat and the New Zealand White rabbit, both of which are widely accepted animal models for teratogenesis studies (Johnson, 1983). Considering logistical constraints of the study and the potential for interspecies cross contamination by endogenous pathogens, the exposure of these two species to OF II will be conducted independently. Therefore,

the study will comprise two major parts. In addition, the project calls for a preliminary, pilot study and an adjunct toxicokinetics study.

Pilot Study

Prior to the initiation of the inhalation study with rabbits, several practice rabbits will be required to perfect the artificial insemination technique in this species. For this purpose eight practice animals will be utilized.

Exposure of Pregnant F-344 Rats to OF II Vapor

The experimental protocol calls for 4 groups of 30 rats each to be exposed to 0.0, 2.4, 24.0 and 240.0 mg/m^3 of OF II vapor. These exposures will be done in Thomas Domes, and the daily exposure duration will be 6 h for a period of 10 consecutive days. The exposure days will correspond to days 6 through 15 of gestation, inclusive. To facilitate conduct of the teratogenesis assay, six animals per day per exposure group will be introduced into the exposure chambers. Thus, the overall time required to complete the exposures will be 14 days. Additional animals for each exposure group will be included for toxicokinetics investigation. Tentatively, the number of additional animals per exposure group is five. These animals will be introduced to the study on exposure day one, and will remain on study until the termination of the exposures.

Beside serving as the exposure chamber, the Thomas Domes will be used for animal housing between exposure periods. Water will be provided to the animals *ad libitum*; however, food will be withheld during the daily exposure period. Animal waste products will be disposed of daily.

During the exposures, a daily gross examination of the animals will be made. Animal weights will be recorded immediately prior to the start of the exposures and at the end of daily exposure on days 3, 6, and 10 of the study. Blood samples will be taken for determination of methemoglobin levels from all animals on study once prior to the initiation of the exposures and at the time of sacrifice. These samples will be analyzed using a Co-oximeter (Model 282, Instrumentation Laboratory).

On days 10 through 14 of the study, rats having undergone 10 consecutive days of exposure will be held for 3 days and then sacrificed. Teratogenesis will be evaluated in fetal tissues, and will include but not be limited to the following examinations: At the time of sacrifice the dam will undergo a macroscopic examination for evidence of structural abnormalities or pathological changes that may have influenced pregnancy. Uterine contents will be examined for embyronic or fetal deaths and the number of potentially viable fetuses counted. The number of corpora lutea will be determined. The fetuses will be weighed and the sex ratio determined. An external gross examination of the fetuses will be conducted, and one third to one half of each litter will undergo

examination for skeletal anomalies. The remaining portion of each litter will undergo evaluation for soft tissue anomalies.

Exposure of Pregnant New Zealand Rabbits to OF II Vapor

In this part of the investigation 4 experimental groups of 15 rabbits each will be exposed to 0.0, 2.4, 24.0, and 240.0 mg/m^3 concentrations of OF II vapor. The length of the daily exposures will be 6 h and exposure duration will be for 13 days corresponding to days 6 - 18 of gestation. The animals will be introduced into the study in increments of five per day per group so as to facilitate the teratogenesis assay. Thus, the total inhalation exposure period will be 15 days. All other aspects of the study will be as previously described for the experiments with rats.

Statistical analyses will be performed on the data gathered in all phases of this investigation, where appropriate. The animals' body weights will be examined using a repeated-measures design analysis. Methemoglobin data will be analyzed by analysis of covariance with the pre-exposure methemoglobin levels as the covariate. Data collected from the teratogenesis assay will be analyzed by a factorial analysis of variance. Statistical analysis methods used will be those of Barcikowski (1983). Experimental bias control will be accomplished by random assignment of animals to the various test groups using a body weight normalized selection process described by Kutzman *et al.* (1986).

OF II Atmosphere Generation, Characterization, and Industrial Hygiene

Generation. The OF II vapor exposure atmospheres will be generated with a thermally controlled, nonrecycling evaporative tower vaporization system similar to one used previously in this laboratory for OF II exposures (Gaworski *et al.*, 1985). Three separate generation systems will be used, one for each of the three OF II exposure levels. These systems differ only in the number of towers employed and the magnitude of several of the operating parameters of the system. Hence, a brief description of one such system will suffice for all. OF II will be pumped at a controlled rate through heated glass evaporating tower(s), and the tower(s) will be purged with a regulated inert carrier gas (N2) flow. The OF II saturated carrier gas will then be metered into the exposure chamber airflow or delivered to a bypass exhaust system. Generator output will be controlled via adjustment of three generator operating parameters, OF II feed rate, carrier gas flow, and temperature of the evaporative tower(s). OF II feed rate to the generator will be adjusted so that, if possible, all the OF II will be evaporated while in residence in the tower(s). Should this not be feasible, then the "generator effluent" OF II will be collected separately for appropriate disposal. Thus, the generators will operate as single-pass systems. All generator systems will be equipped with automatic emergency controls including OF II feed, carrier gas feed shutdowns, and thermal excursion

regulators. Generator output lines will have chamber bypass lines connected directly to the exhaust system lines as an additional level of emergency control.

Characterization. Of II concentration in the exposure chambers will be measured by continuous real-time monitoring of PGDN concentration, using infrared (IR) spectroscopy. Four Miran 1A IR spectrometers, one for each exposure concentration, will be calibrated and standardized with PGDN purified from generator OF II prior to the exposures. The concentration and stability of the three constituents in OF II will be determined chromatographically prior to and upon termination of each inhalation study. If possible, the nominal concentration of OF II in the exposure environment will be determined to adhere to regulatory guidelines. Contingent upon receipt of the appropriate instrumentation, the exposure chambers will be monitored for the formation of OF II condensation aerosols.

Industrial Hygiene. Environmental monitoring of OF II vapor will be continuous using gas chromatography. Positive control analysis will be provided by sampling the low-concentration exposure atmosphere.

REFERENCES

Barcikowski, R.S. 1983. *Computer Packages and Design.* Vol. 1, BMDP. Lanham, MD: University Press of America.

EPA Health Effects Test Guidelines. 1982. Office of Pesticides and Toxic Substances, Office of Toxic Substances (TS-792). EPA 560/6-82-001. Washington, DC: U.S. Environmental Protection Agency.

Gaworski, C.L., J. D. McEwen, E.H. Vernot, C.C. Haun, H.F. Leahy, and W.J. Bashe. 1985. A one year inhalation study of Otto Fuel II. AAMRL-TR-85-071 (AD A-163162), pp. 1-27. Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory.

Johnson, E.M. 1983. Current status of, and conditions for, estimation of risk to the human conceptus from environmental chemicals. In: M.S. Christian, W.M. Galbraith, P. Voytek, and M.A. Mehlman, eds. *Assessment of Reproductive and Teratogenic Hazards,* pp. 99-116. Princeton, NJ: Princeton Scientific Publishers, Inc.

Jones, R.A., J. A. Strickland, and J. Seigel. 1972. Toxicity of propylene glycol 1,2-dinitrate in experimental animals. *Toxicol. Appl. Pharmacol.* 22:128-137.

Kutzman, R.S., R.T. Drew, and R.N. Shiotsuka. 1986. Pulmonary changes resulting from subchronic exposure to cadmium chloride aerosol. *J. Toxicol. Environ. Health* 17:175-189.

Stewart, R.D., J.E. Peterson, P.E. Newton, C.L. Hake, M.J. Hosko, A.J. Lebrun, and G.M. Lawton. 1974. Experimental human exposure to propylene dinitrate. *Toxicol. Appl. Pharmacol.* 30:377-395.

U.S. Navy Environmental Health Bulletin. 1976. *NAVMED P-5112.*

3.3.4 Toxicological Evaluations of Triethylborane (TEB)

A. Vinegar, E. R. Kinkead, E. C. Kimmel, R. B. Conolly, and H. F. Leahy

INTRODUCTION

Triethylborane (TEB) is a colorless liquid that ignites upon exposure to air. The vapor of TEB can exist in air at concentrations below 1300 ppm without igniting. TEB is used as a flare and to cause ignition of fuels. Its combustion products are boron-containing particles, probably boric acid or boron oxide and ethanol.

There are no published data on the toxicity of TEB in humans. Unpublished clinical reports indicate both CNS and respiratory symptoms after accidental exposure. Some published data exist for exposures to diborane, pentaborane, and decaborane. Pulmonary symptoms are more evident after diborane exposures, and CNS symptoms more evident after pentaborane and decaborane exposures (Cordasco *et al.*, 1962; Lowe and Freeman, 1957). Hart *et al.* (1984) studied 14 individuals who had been accidentally exposed to pentaborane. Using neuropsychological tests and personality inventories, they found electroencephalographic changes consistent with toxic encephalopathy; increases in depression and anxiety; and impairment of attention, recent memory, and problem-solving ability that might be attributed to emotional changes.

The only information available on TEB toxicity is contained in a report by Rinehart (1960). Groups of five or six male rats of nonspecified strain, each weighing 200 to 300 g, were exposed to varying doses of TEB as a vapor for single 4-h periods or as a solution in mineral oil administered intragastrically (i.g.) or intraperitoneally (i.p.). The 4-h LC_{50} for the vapor was 700 ppm. The LD_{50}s from the i.g. and i.p. studies were 235 mg/kg and 22.7 mg/kg, respectively. Respiratory irritation was apparent in the inhalation studies, and CNS effects were evident in the i.g. and i.p. studies. No histopathology was done. The chamber concentrations in the inhalation studies were nominal; they were not sampled and analyzed. Extrapolations between routes of exposure were poor because blood concentrations were not measured.

Setting exposure guidelines is difficult based on the incomplete information available for TEB. Therefore, we propose the following studies to gather more complete and meaningful data to allow assessment of exposure limits in man.

MATERIALS AND METHODS

Chemical Analysis

Infrared and gas chromatographic methods are under development for analyzing TEB in the vapor phase and in the blood and/or urine of exposed animals. These methods will be employed to monitor inhalation exposure concentrations as well as to measure TEB body burdens and excretion rates.

Vapor Phase

An IR spectrum of a commercially available mixture of hexane and TEB was determined using a thin layer of fluid between salt plates. This was compared with the spectrum of mixed hexanes. There was an absorption peak in the region of 7.5 μ that appears to be due to the presence of TEB.

The Miran 1A will be used at 7.5 μ and calibrated with bags containing TEB in nitrogen. The dilution nitrogen will be the baseline for measuring the absorption band of TEB.

Biological Sampling

The analysis of biological tissues for presence of boron itself may give no useful information as to the presence of TEB in the system. There are few methods available other than adaptations of analytical techniques using soil or plant tissue. Animal levels are rarely reported, are at the limit of sensitivity of wet chemistry methods, and have been inconsistent.

TEB is not very soluble in water, but it is quite soluble in organic solvents. Therefore, the analysis of TEB in biological fluids may be carried out using a simple hexane extraction followed by gas chromatographic separation and flame ionization detection. TEB has been successfully separated chromatographically using a 50-meter-long SE-54 capillary column. Further work on this method will be required to describe conditions needed for the desired maximum sensitivity.

Inhalation Toxicity

Groups of 10 male and 10 female Fischer-344 rats will be exposed once to TEB vapor for 4 h. At least three levels of exposure concentration will be used, spaced appropriately to produce a range of toxic effects and mortality rates sufficient to permit the calculation of an LC_{50}. Similar groups of animals exposed to air only will serve as controls. Body weights, clinical signs, mortality, and histopathology will be assessed. Clinical chemistry (including liver function tests), hematology, and TEB blood levels will be determined for surviving animals.

Pulmonary Function

Lung function will be assessed on three groups of 10 male and 10 female Fischer-344 rats: (1) survivors from the LC_{50} concentration of TEB immediately after exposure and at later time periods, (2) individuals exposed to a no-effect level of TEB, and (3) controls exposed to air only.

Animals will be anesthetized with halothane and transorally intubated. The following measurements will be made using plethysmographic techniques: tidal volume flow and transpulmonary pressure from which dynamic compliance and pulmonary resistance will be calculated; quasistatic volume-pressure curves from which static compliance and vital capacity will be determined; maximum expiratory flow-volume curves from which flows at 75, 50, and 25% of vital capacity can be derived; and DLco for evaluating gas diffusion across the lung.

Airway Reactivity

Changes in airway reactivity will be assessed by determining dose-response curves of acetylcholine delivered i.v. during plethysmographic measurement of specific airway conductance in awake English short-haired guinea pigs both before and after exposure to TEB. The exposure regime will be the same as described for the rats under Pulmonary Function.

Behavioral Studies

Two types of behavioral tests, the startle test and the figure-eight maze will be used to evaluate neurotoxicity. Startle represents a short-latency reflex mediated by a simple neural circuit that exhibits habituation, sensitization, prepulse modification, and enhancement by startle after classical conditioning. It is affected by a number of drugs and is a good model for studying how neurotransmitters modulate sensory-motor reactivity (Davis, 1980). The figure-eight maze measures motor activity, which reflects general neurological status of the organism. Charges brought about by a variety of neurotoxic mechanisms result in changes in motor activity (Reiter, 1983).

Pharmacokinetics

Pharmacokinetic modeling techniques will be used to allow prediction of distribution of TEB in body compartments. Data obtained will allow extrapolation to human exposures.

REFERENCES

Cordasco, E.M., R.W. Cooper, J.V. Murphy, and C. Anderson. 1962. Pulmonary aspects of some toxic experimental space fuels. *Diseases of the Chest* 41:68-74.

Davis, M. 1980. Neurochemical modulation of sensory-motor reactivity: Acoustic and tactile startle reflexes. *Neurosci. Biobehav. Rev.* 4:241-263.

Hart, R.P., J.J. Silverman, L.K. Garrettson, C. Schulz, and R.M. Hamer. 1984. Neuropsychological function following mild exposure to pentaborane. *Am. J. Indust. Med.* 6:37-44.

Lowe, H.J. and G. Freeman. 1957. Boron hydride (borane) intoxication in man. *A.M.A. Arch. Ind. Health* 16:523-533.

Reiter, L.W. 1983. Chemical exposures and animal activity: Utility of the figure 8 maze. In: A.W. Hayes, R.C. Schnell, and T.S. Miya, eds. *Developments in the Science and Practice of Toxicology*, pp. 73-84, Elsevier.

Rinehart, W.E. 1960. Studies on the toxicity of triethylborane (TEB). *Am. Ind. Hyg. Assoc. J.* 21:389-393.

3.3.5 The Use of Cell Culture to Compare Liver and Kidney Metabolites of 2,3,4-Trimethylpentane

N.J. Del Raso

INTRODUCTION

Studies conducted at AAMRL have shown sex differences in the response of rats exposed to the vapors of hydrocarbon propellants such as JP-4 and JP-5 (complex mixtures of aliphatic and aromatic hydrocarbons; Bruner and Pitts, 1983). Kidney lesions occur only in male rats after exposure to hydrocarbon propellant vapors. These lesions consist of greatly increased cytoplasmic hyaline droplets in the epithelial cells of the proximal tubule, necrosis of proximal tubular cells, and intratubular blockage by cell debris at the junction of the outer and inner stripe of the outer medulla (Bruner, 1984). Long-term exposure of male rats to these hydrocarbon vapors results in abundant mineralized casts in the medullary tubules and a significant increase in the incidence of renal tumors (Bruner, 1984). Studies investigating the toxic and carcinogenic effects of "pure" hydrocarbon propellants such as 2,2,4- and 2,3,4-trimethylpentane (TMP), RJ-4 propellant, and JP-IO, in most cases, have resulted in kidney lesions in male rats similar to those produced by hydrocarbon mixtures (Bruner and Pitts, 1983). The pathogenesis of hydrocarbon nephropathy must be characterized in order to assess the risks to USAF personnel working with hydrocarbon propellants.

In an attempt to characterize the pathogenesis of hydrocarbon propellants, 2,3,4-TMP, a principal constituent of gasoline involved in assigning the octane rating, has been used in gavage studies. A single gavage dose of 2,3,4-TMP was found to produce hydrocarbon nephropathy in male Fischer-344 rats (Hobson *et al.*, 1985). The major metabolites of 2,2,4-TMP metabolism by the kidneys of male rats have been identified in urine (Olson *et al.*, 1985). The metabolic pathway of 2,2,4-TMP by the male rat kidney has been theoretically described, but the metabolism by the liver has not yet been investigated. The production of alpha-2u-globulin, a low molecular weight (MW 26,400) protein, in the liver of male rats is influenced by testosterone and other interactive hormones (Irwin *et al.*, 1971). This protein is filtered by the kidney through the glomeruli, and is reported to be the major urinary protein in male rats. Alpha-2u-globulin is not appreciably synthesized by the liver of normal female rats or other species (Roy, 1973). Therefore, alpha-2u-globulin is believed to be the major constituent of the cytoplasmic hyaline droplets seen in epithelial cells of the proximal tubule of male rats exposed to hydrocarbon vapors, and evidence for this has been reported (Alden *et al.*, 1983). Factors resulting in the excessive accumulation of alpha-2u-globulin in the proximal tubular cells may be responsible for the pathogenesis of hydrocarbon nephropathy. It is possible that TMP and/or its metabolites are inhibiting proximal tubular lysosomal enzymes, or other catabolic

proteins, preventing the degradation of alpha-2u-globulin. Therefore, it is necessary to compare the metabolites of TMP metabolism by the kidney with that by the liver.

The objective of this study is to isolate and establish primary cultures of male rat proximal convoluted tubular and hepatic cells suitable for experimental exposure to sublethal concentrations of 2,3,4-TMP. The viability of isolated cells will be measured to determine satisfactory isolation procedures. Using cell lysis as a measure of toxicity, dose-response curves will be determined for 2,3,4-TMP in order to establish suitable exposure doses for metabolic studies. The appropriate exposure of kidney and liver cells to 2,3,4-TMP for metabolic studies will be implemented and the samples analyzed by the Air Force. Electron microscopic (EM) verification of cell types and EM studies of morphology of 2,3,4-TMP exposed cells will also be conducted by the Air Force.

MATERIALS AND METHODS

Kidney Tubule Cell Isolation

Kidneys will be excised from adult rats and decapsulated. The corticies will be dissected, minced with scissors to approximately 2-cm pieces, and washed three times in ice-cold Joklik's medium. The tissue will then be digested by Type II collagenase (0.2 mg/mL), Type V hyaluronidase (0.3 mg/mL) in calcium, and magnesium-containing minimal essential medium (MEM) with Earle's salts (5 g of wet tissue to 50 mL of digestion mixture) for 1 h at 37°C in a Teflon-stirred digestion vessel. Nephron components will be released by gentle shear, by syringing three times through a 50-mL syringe with a 19-gauge needle into a 50-mL conical centrifuge tube. The cell mixture will then be filtered through a 62-μ nylon mesh into a sterile 100-mL beaker. One to two milliliters of the cell mixture will then be sedimented at 1 x g in 10-mL of ice-cold Joklik's medium in sterile, 15-mL conical centrifuge tubes. The final material will be resuspended in Dulbecco's minimal essential medium (DMEM) and designated as the pool fraction. An aliquot of the pool fraction will then be removed and a viable cell count determined by trypan blue (0.04%) exclusion with a hemacytometer.

Discontinuous gradients of Percoll will be used for fractionation of pool material. Percoll consists of colloidal silica particles of 15 to 30 nm diameter that have been coated with polyvinylpyrronol for increased stability and decreased toxicity. Sterile stock isosmotic Percoll solutions (SIP) will be made by adding Percoll to 10X DMEM 9:1 by volume. The pool fraction will be adjusted with DMEM to give a total of 10 to 40 million cells in 1 to 2 mL of cell suspension. A discontinuous Percoll gradient will be made by layering 6 mL of SIP (90% Percoll) on the bottom of a 17- x 100-mm Falcon round bottom tube, followed by 6 mL of a homogeneous mixture containing 5 mL of a 41.9% Percoll solution and 1 mL of the adjusted pool fraction, and finally adding 1 mL of Dulbecco's phosphate buffer solution (DPBS). The tubes will be centrifuged at 40 x g for 10 min at room temperature. The two resulting fractions, the 0-41.9% interface fraction (1) and the 41.9%-

90% interface fraction (2), will then be collected. Fraction 1 will be enriched with glomeruli and distal segments, while fraction 2 will be enriched with proximal tubular segments.

Cell Culture

Suspension cultures of kidney tubule cells will be made by isolating fraction 2 from the Percoll gradients and resuspending the cells at a concentration of approximately 400,000 cells/mL with Eagle's MEM supplemented with nonessential amino acids, glutamine (292 µg/mL), penicillin (100 µg/mL), streptomycin (100 µg/mL), and fetal bovine serum (10% v/v). Four milliliters of the resultant cell suspension will then be added to 25 mL siliconized glass Erlenmeyer flasks and incubated at 37°C (95% oxygen: 5% carbon dioxide) on a clinical rotator at 90 rpm. Cell culture procedures for the kidney are based on Scholer and Edelman (1979), Richardson and Simmons (1980), and Richardson *et al.* (1982).

Liver Perfusion and Hepatocyte Isolation

Hank's balanced salt solution (HBSS; calcium and magnesium free, without phenol red) will be supplemented with bovine serum albumin (500 µg/mL; Fraction V), penicillin (100 µg/mL), streptomycin (100 µg/mL), magnesium sulfate (174 µg/mL), heparin (2.0 U/mL), insulin (0.5 µg/mL), and sodium bicarbonate (2.2 mg/mL). This perfusion medium will be sterilized by filtration (0.45-µ pore size) and continuously bubbled with a 95% oxygen:5% carbon dioxide mixture (resulting pH 7.4). The medium will be maintained at 39°C, allowing for two degrees drop in temperature while traversing the perfusion tubing.

A 250 to 350 g rat will be anesthetized by an intraperitoneal injection of a mixture of ketamine (70 mg/mL) and xylazine (6 mg/mL) at 1 mL/kg body weight. The rat will be placed on its dorsum on a restraining board with its front and hind legs secured. All subsequent procedures will be implemented aseptically. After making a verticle cut down the abdomen of the rat through the subcutaneous tissue, the opening of the abdominal cavity will be completed carefully by cutting through the muscular layer. The connective tissue and fat from around the posterior vena cava, between the renal vein and the liver, will be removed. The posterior vena cava will then be cannulated with a 21-gauge butterfly needle, anterior to the renal vein. After securing the needle with thread, the chest cavity will be opened and the posterior vena cava will be clamped anterior to the diaphragm with a curved hemostat. The perfusion pump will then be activated immediately to force the perfusion medium, containing 2.0 U/mL heparin, through the liver. After 10 to 15 s, the hepatic portal vein will be cut to allow the perfusion medium to drain before the pressure within the liver becomes too great. Periodically during the perfusion, medium will be poured over the liver to keep it moist and to help maintain near body temperature. After 10 min, the perfusion medium will be changed to a medium containing 0.5 mg/mL collagenase (Type IV), without heparin. After a

15-min perfusion with collagenase, the lobes of the liver will be removed, cut into 2- to 3-mm pieces, and placed into 40 mL of perfusion medium at 4°C in a sterile 50-mL beaker. Single cells will then be expelled from the liver pieces by stirring with a Pasteur pipette while simultaneously bubbling the mixture with 95% oxygen:5% carbon dioxide. This will be repeated with at least three changes of medium. The stromal tissue will then be removed by successive filtrations through 250-μ and 62-μ pore-size monofilament nylon meshes housed in 150-mL capacity filtering units (gamma irradiated). The cells will then be transferred to a 50-mL centrifuge tube and washed two times by centrifugation for 3 min at 50 x g in calcium-free HBSS.

Hepatocyte Enrichment and Culture

Leibovitz L-15 tissue culture medium (L-15A) will be supplemented with the following: HEPES (15 mM), penicillin (100 μg/mL), streptomycin sulfate (100 μg/mL), glucose (1.5 mg/mL), insulin (0.5 μg/mL), and sodium bicarbonate (2.2 mg/mL). The resulting pH will be 7.4. Leibovit L-15 tissue culture medium (L-158) will be supplemented with HEPES (18 mM), penicillin (100 μg/mL), streptomycin sulfate (100 μg/mL), glucose (1.5 mg/mL), insulin (0.5 μg/mL), sodium bicarbonate (2.2 mg/mL), and bovine serum albumin (2.0 mg/mL; Fraction V). The resulting pH will be 7.4. Media will be maintained at 4°C.

A sterile SIP solution will be made by adding nine volumes of stock Percoll to one volume of 10X HBSS. Twenty-four-milliliter aliquots of the SIP solution will be added to sterile 50-mL conical-capped centrifuge tubes and stored at 4°C until needed (two-month shelf life). Isolated hepatocytes will be washed 2X by centrifugation in calcium-free HBSS for 3 min at 50 x g. The cell concentration will then be adjusted to between 5.0 and 10.0 million cells/mL with L-15B medium. Twenty-five-milliliter aliquots of the cell suspension will then be distributed to 50-mL centrifuge tubes containing the 24-mL aliquots of the SIP solution. The tubes will be capped and gently inverted five times to ensure a homogeneous suspension. This will produce a medium of a density of 1.06 g/mL. The cell suspensions will be centrifuged at 50 x g for 10 min at 4°C. The viable parenchymal cells will be in pellets at the bottoms of the tubes. The supernatant will consist of a flocculent layer (0.5- to 1.0-cm thick) of nonparenchymal cells, nonviable cells, cell aggregates and debris. A clear layer (5- to 6-cm thick) will lie between the flocculent layer and the viable parenchymal cells. After carefully aspirating the supernatant, the hepatocytes will be resuspended in 25 mL of the L-15B culture medium and washed 2X by centrifugation at 50 x g for 2 min.

After washing 2X, the hepatocytes will be resuspended at a concentration of approximately 400,000 cells/mL in L-15A culture medium. Four milliliters of the resultant cell suspension will then be added to 25-mL siliconized glass Erlenmeyer flasks and incubated at 37°C (95% oxygen:5% carbon dioxide) for 24 h on a clinical rotator at 90 rpm. Liver procedures are based on Berry and Friend

(1969), Michalopoulus and Pitot (1975), Michalopoulus *et al.* (1978), Oldam *et al.* (1979), and Kreamer *et al.* (1986).

REFERENCES

Alden, C.L., R.L. Kanerva, L.C. Stone, and G. Ridder. 1983. The pathogenesis of the nephrotoxicity of volatile hydrocarbons in the male rat. *Proc. Workshop on the Kidney Effects of Hydrocarbons.* American Petroleum Institute, Boston, MA.

Berry, M.N. and D.S. Friend. 1969. High-yield preparation of isolated rat liver parenchymal cells. *J. Cell Biol.* 43: 506-520.

Bruner, R.H. 1984. In: M.A. Mehlman *et al.*, eds. *Renal Effects of Petroleum Hydrocarbons*, pp. 133-140. Princeton, NY: Princeton Scientific Publishers, Inc.

Bruner, R.H. and L.L. Pitts. 1983. Nephrotoxicity of hydrocarbon propellants to male Fischer-344 rats. *Proc. 13th Annual Conf. Environ. Toxicol.*, pp. 337-349. Wright-Patterson AFB, OH: Air Force Aerospace Medical Research Laboratory.

Hobson, D.W., A.P. D'Addario, D.E. Uddin, C.T. Olson, and M.P. Serve. 1985. Use of a rapid urine screening procedure to determine the relative nephrotoxicity of trimethylpentane isomers in the male F-344 rat. *The Toxicologist* 5(1):58.

Irwin, J.F., S.E. Lane, and O.W. Neuhaus. 1971. Synthetic effect of glucocorticoids and androgens on the biosynthesis of a sex-dependent protein in the male rat. *Biochem. Biophys. Acta* 252:328-334.

Kreamer, B.L., J.L. Staecker, N. Sawada, G.L. Satter, M.T. Stephen Hsia, and H.C. Pitot. 1986. Use of a low-speed, iso-density percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. *In Vitro Cellular and Develop. Biol.* 22:201-211.

Michalopoulus, G., G.L. Sattler, and H.C. Pitot. 1978. Hormonal regulation and the effects of glucose on tyrosine aminotransferase activity in adult rat hepatocytes cultured on floating collagen membranes. *Cancer Res.* 38:1550-1555.

Michalopoulus, G. and H.C. Pitot. 1975. Primary culture of parenchymal liver cells on collagen membranes. *Exp. Cell Res.* 94:70-78.

Oldam, J.W., D.A. Casciano, and J.A. Farr. 1979. The isolation and primary culture of viable, nonproliferating rat hepatocytes. *TCA Manual* 5(2):1047-1050.

Olson, C.T., K.O. Yu, D.W. Hobson, and M.P. Serve. 1985. Identification of urinary metabolites of the nephrotoxic hydrocarbon 2,2,4-trimethylpentane in male rats. *Biochem. Biophys. Res. Commun.* 130:313-316.

Richardson, J.C., P. Waterson, and N.L. Simmons. 1982. Isolation and culture of renal cortical tubules from neonate rabbit kidneys. *Q. J. Exp. Phys.* 67:287-301.

Richardson, J.C. and N.L. Simmons. 1980. A rapid method for fractionation of nephron components using a non-toxic isosmotic gradient material (Percoll). *Proc. Phys. Soc.* 12:1-2P.

Roy, A.K. 1973. Androgen-dependent synthesis of alpha-2u-globulin in the rat: Role of the pituitary gland. *J. Endocrinol.* 56:295-301.

Scholer, D.W. and I.S. Edelman. 1979. Isolation of rat kidney cortical tubules enriched in proximal and distal segments. *American J. Phys.* 237:F350-359.

3.4 INSTALLATION RESTORATION PROGRAM

3.4.1 Evaluation of the Acute Toxicity of Selected Groundwater Contaminants

E. R. Kinkead, H. F. Leahy

INTRODUCTION

The THRU was requested to evaluate the acute toxicity of several groundwater contaminants. These were chosen from a list of groundwater contaminants reviewed for the Air Force by the A.D. Little Company (1985). Hazard evaluations of these compounds could not be completed because insufficient acute toxicity data were available. The final risk assessments with these chemicals will be conducted based on principles and considerations derived from a physiologically based, pharmacokinetic modeling approach. These studies provide one arm of the risk assessment, the definition of the dose-response relationship for an appropriate toxic response. Additional toxicity data were lacking for tetrachloroethylene, (TRCE) 1,1,1-trichloroethane, chlorobenzene, and 1,1-dichloroethylene, and acute inhalation toxicity data were lacking for 2,3-dimethylphenol (DMP) and 1,1-dichloroethane.

MATERIALS AND METHODS

Animals

Male, albino, New Zealand White rabbits, weighing between 2 and 3 kg, and male Fischer-344 rats, weighing between 200 and 250 g, were used in these studies. The animals were maintained on a photoperiod of 12 h light (beginning at 0600 hours) and 12 h darkness. Food and water were available *ad libitum*. Quality control studies conducted by Air Force personnel during the quarantine period showed the animals to be in good health.

Test Compounds

Table 3.4.1-1 describes the compounds tested in this study.

Quality control analyses were performed on all six compounds. The five chlorinated compounds were analyzed using a gas chromatograph (GC), which was equipped with a thermal conductivity detector to allow a better comparison of peak area/mass for percent-purity calculations in mixtures possibly containing both halogenated and nonhalogenated hydrocarbons. The compound 2,3-dimethylphenol (DMP), a solid, was listed by the supplier as 97% pure. However, a headspace vapor sample revealed a number of volatile-compound impurities. The concentration of volatile compounds was reduced to below the level of sensitivity by metering air through a column containing DMP for 45 min. Because the inhalation test was to be conducted to specifically gauge

the effects of DMP, this method was used to remove the impurities from the test sample prior to conducting the inhalation exposure. The purified DMP was then analyzed using a GC equipped with a flame ionization detector. Approximately 0.05 mg of the solid material was injected into the GC using a syringe with a side cavitation. Table 3.4.1-2 shows the purity reported by the suppliers, as well as the purity determined chromatographically.

TABLE 3.4.1-1. TEST COMPOUNDS

Compound	CAS No.	Density	Formula Weight	Route of Testing
Tetrachloroethylene[a] (TRCE)	127-18-4	1.614	165.83	Dermal
1,1,1-Trichloroethane[a]	71-55-6	1.100	133.4	Dermal
Chlorobenzene[a]	108-90-7	1.100	112.56	Dermal
1,1-Dichloroethylene[b]	75-34-4	1.213	96.94	Dermal
2,3-Dimethylphenol[b] (DMP)	526-75-0	–	122.17	Inhalation
1,1-Dichloroethane[b,c]	75-34-3	1.177	98.96	Inhalation

[a] Purchased from J.T. Baker Chemical Co.
[b] Purchased from Aldrich Chemical Co.
[c] Contained approximately 3% dioxane as a stabilizer

TABLE 3.4.1-2. COMPARISON BETWEEN THE REPORTED PURITY AND MEASURED PURITY OF COMPOUNDS USED IN GROUNDWATER STUDY

	Purity (%)	
Compound	Reported	Measured
Tetrachloroethylene (TRCE)	100.0	100.0
1,1,1-Trichloroethane	99.9	96.4
Chlorobenzene	99.9	99.9
1,1-Dichloroethylene	99.0	100.0
2,3-Dimethylphenol (DMP)	97.0	98.1
1,1-Dichloroethane	[a]	92.6

[a] No purity analysis provided by vendor.

Dermal Toxicity Testing

Testing was initiated by dosing five rabbits with 2 mL test material/kg body weight. If there was no mortality at this level no additional dose levels would be tested. If mortality had been produced, testing would have continued with five animals per dose level.

Each rabbit's fur was shaved as closely as possible with an Oster® clipper equipped with surgical blades and a vacuum attachment. The rabbits' back and sides (about halfway down to the abdomen) were clipped from the saddle area of the shoulders to the top of the rear leg area. The animals were individually weighed prior to dosing to determine the proper dose volume. Undiluted liquid material was applied to the back of the rabbit and in equal portions to the two sides. The

compound was kept in place with 8-ply gauze patches. Clear plastic wrap was then applied to the entire clipped back area, and the entire midsection of the rabbit was wrapped with Elastoplast® tape. After a 24-h exposure to the compound, the tape, plastic wrap, and gauze were removed. The rabbits were housed in individual cages and observed for mortality or other signs of toxicity frequently on the day of dosing and twice daily thereafter. Clinical signs were recorded on symptomatology forms. Body weights were obtained at the time of dosing and on days 1, 2, 4, 7, 10, and 14 posttreatment.

In addition to the standard dermal test, blood levels of tetrachloroethylene (TRCE) were measured during and after the 24-h dermal contact. Five rabbits were exposed to the upper dosing limit of 2 mL/kg. Blood samples were taken from the rabbits' ears on an alternating schedule such that the same ear was never used for consecutive samplings. Samples were taken at 2, 4, 8, and 24 h during contact and at 4, 8, and 24 h and 2 and 7 days following exposure. To lessen anxiety during blood collection, rabbits were lightly sedated with an intramuscular injection of Innovar-Vet® (0.15 mL/kg).

Inhalation Testing

Five male Fischer-344 rats weighing between 200 and 250 g were exposed to saturated vapors of DMP for 4 h in a clear, 120-L Plexiglas chamber. The concentration of DMP in the chamber was controlled by metering air through a column packed with pulverized DMP, and the chamber atmosphere was monitored until maximum concentration was achieved. The rats were then placed into the exposure chamber via a cage-drawer system that allows insertion of the cage with minimal loss of contaminant. Following the 4-h exposure, the rats were observed for 14 days. The rats were weighed at 1, 2, 4, 7, and 14 days postexposure, and the body weights were compared to those of control animals.

Four groups of five male Fischer-344 rats weighing between 195 and 262 g were exposed to several different concentrations of 1,1-dichloroethane for 4 h. This liquid agent was vaporized by metering it into an evaporator. A controlled airflow, which passed through the evaporator at a minimum of 28 L/min (20 chamber volumes/h), carried the vapor to a 60-L exposure chamber. The rats were observed during exposure and twice daily for 14 days. Body weights were obtained prior to exposure and at 1, 2, 3, 7, and 14 days postexposure. The LC_{50} of 1,1-dichloroethane was calculated from the number of deaths that occurred during the 14-day postexposure period using the moving average method of Weil (1952). Gross examinations of lung, liver, spleen, and brain were performed on each rat sacrificed at 14 days posttreatment.

Analysis of Exposure Atmospheres

A Miran 1A infrared analyzer (Foxboro) was used to monitor the concentration stability of DMP in the chamber, while the actual concentration of DMP was determined by GC analysis of impinger samples collected in water. The GC detector peak areas were compared to DMP standards in water.

The 1,1-dichloroethane concentration of the chamber was monitored continuously with a Miran 1A infrared analyzer equipped with an 8-cm gas cell. The absorbance data were converted to part-per-million values using linear regression calibration plots that were established using known atmospheric concentrations prepared in 5-L Mylar® bags. The exposure concentrations for individual studies were determined using standard absorbance data that closely bracketed the upper and lower ends of the scale of those observed during the exposure.

Blood Analysis

Blood samples were taken from dermally exposed rabbits for assessment of TRCE levels. The presence of TRCE in the rabbits' blood was determined following extraction of TRCE from the blood with hexane. This extract was analyzed using a GC equipped with an electron capture detector. Therefore, the reported blood concentration results reflect extractable rather than total TRCE.

A standard curve was generated by comparing the area under the gas chromatogram curves to the concentration of TRCE in the extract. Test-data peak areas were converted to blood concentrations using a linear least-squares analysis over limited ranges of the standard curve that corresponded to, and bracketed, the test data. These conversions were also investigated by polynomial least-squares analysis, employing all of the standard data. No significant differences were observed in the blood concentration data generated using these two methods. All of the data presented were generated using the linear method.

Dermal Exposure

Dermal toxicity tests were conducted at a maximum upper dose limit of 2 mL/kg. None of the four compounds caused mortality. After an initial reduction in body weight, all rabbits demonstrated weight gains. A summary of the dermal toxicity data is provided in Table 3.4.1-3.

Five rabbits were dermally exposed to 2 mL/kg of TRCE for 24 h. Serial blood samples were taken for TRCE determinations at various time periods throughout 7 days following the exposure. None of the rabbits died as a result of the TRCE exposure. One rabbit sustained an injury and had to be euthanized after the 4-h posttreatment blood sample was taken. The results of the blood analyses are provided in Table 3.4.1-4. Preexposure or control rabbit blood values were not determined

during these studies. However, a sample of blood drawn from an untreated rabbit before this study began did not reveal a peak at the retention time characteristic of TRCE.

TABLE 3.4.1-3. RESULTS OF ACUTE DERMAL TOXICITY TESTING OF SELECTED GROUNDWATER CONTAMINANTS ON MALE RABBITS DOSED WITH 2mL/kg

Compound	14-Day Mortality Ratio
Tetrachloroethylene (TRCE)	0/5
1,1,1-Trichloroethane	0/5
Chlorobenzene	0/5
1,1-Dichloroethylene	0/5

TABLE 3.4.1-4. TETRACHLOROETHYLENE CONCENTRATIONS IN RABBIT BLOOD DURING AND AFTER DERMAL CONTACT OF 2 mL/kg

	Sampling Time	TRCE µg/mL Mean (N = 5) ± S.D.
During Exposure	2 h	2.361 ± 0.844
	4 h	1.107 ± 0.323
	8 h	0.693 ± 0.285
	24 h	0.243 ± 0.139
Postexposure	4 h	0.304 ± 0.088
	8 h	0.354[a] ± 0.147
	24 h	0.110[a] ± 0.020
	2 days	0.083[a] ± 0.045
	7 days	0.015[a] ± 0.007

[a] N = 4

Inhalation Exposure

Five male Fischer-344 rats were exposed by inhalation to a mean concentration of 85.5 mg/m^3 DMP for 4 h. None of the rats died during the exposure or during the 14-day observation period. Mean gains in body weight of the exposed rats during the postexposure period compared favorably with the weight gains of the control rats. Gross pathologic examination of the rats following the 14-day postexposure period revealed no exposure-related lesions.

Four groups of five rats each were exposed to various concentrations of 1,1-dichloroethane for 4-h periods. The concentrations and mortality data are provided in Table 3.4.1-5. All rats were rapidly anesthetized by 1,1-dichloroethane exposure at each concentration tested. All of the recorded deaths occurred during the exposure period. All rats that survived the 4-h exposure period also survived through the 14-day observation period. Gross pathological examination of the rats that died following exposure revealed congested lungs and livers. Examination of the rats that

survived revealed no exposure-related lesions. An LC_{50} of approximately 13,000 ppm was calculated from the mortality data using the moving average method of Weil (1952). Confidence limits were not calculated because all exposures resulted in either zero or 100% mortality.

TABLE 3.4.1-5. MORTALITY OF MALE RATS AFTER FOUR-HOUR INHALATION EXPOSURES TO 1,1-DICHLOROETHANE

Concentration (ppm)	Mortality Ratio (N = 5)
23,886	5/5
18,353[a]	5/5
17,214	0/5
9,298[a]	0/5

[a] Concentrations rounded to 9,200 and 18,400 for calculation of LC_{50} by the Weil method.

DISCUSSION

The four compounds tested for dermal toxicity (at an upper limit of 2 ml/kg) caused no mortality following 24 h of contact and a 14-day observation period. These compounds would be considered to be of a low order of toxicity by this route.

TCRE was rapidly absorbed and excreted (largely by the lungs; Pegg *et al.*, 1979) within the first eight hours of the 24-h contact period. Very little of the TRCE remained in the blood after 24 h.

DMP proved to be nontoxic after a 4-h inhalation of essentially saturated vapors. No signs of toxic stress were noted in the rats either during or following exposure.

Four-hour inhalation exposures of rats to 1,1-dichloroethane resulted in an LC_{50} of approximately 13,000 ppm. These data are in agreement with a report by Smyth (1956), which stated that rats survived 8 h after a 400-ppm inhalation exposure, but died after receiving a 16,000-ppm dose.

REFERENCES

Arthur D. Little, Inc. 1985. *The Installation Restoration Program Toxicology Guide* Vol. I. DT14NTOSH: ADA 171095. Wright-Patterson Air Force Base, OH: Harry G. Armstrong Aerospace Medical Laboratory

Pegg, D.G., J.A. Zempel, W.H. Braun, and P.G. Watanabe. 1979. Disposition of tetrachloro[^{14}C]ethylene following oral and inhalation exposure in rats. *Toxicol. Appl. Pharmacol.* 51:464-474.

Smyth, H.F., Jr. 1956. Improved communication – hygienic standards for daily inhalation. *Am. Ind. Hyg. Assoc. Q.* 17:129.

Weil, C.S. 1952. Tables for convenient calculation of median-effective dose (LD_{50} or ED_{50}) and instruction in their use. *Biometrics* 8:249-263.

3.4.2 Protocol for the Acute Toxicity Testing of Installation Restoration Program (IRP) Chemicals

E. R. Kinkead

INTRODUCTION

The Air Force is conducting a large-scale program to identify, characterize, and manage hazardous waste sites at Air Force installations worldwide. Although long-term monitoring, risk assessment, and abatement efforts are included, many installations have short-term, site-specific problems requiring immediate abatement action, e.g., open discharges of landfill leachate to areas with public access.

The purpose of this study is to conduct acute and subchronic toxicity testing on a series of leachate samples collected at multiple discharge points under various conditions. The Government will split the samples and conduct chemical analyses. The acute testing will include oral and dermal toxicity, eye and skin irritation, and sensitization. The repeated-dose testing will consist of 14-day oral and 14-day dermal studies. The studies will be conducted following EPA guidelines. Existing alternative methods to animal testing are inadequate for this study.

Results of these toxicity studies along with chemical analyses, will be carefully interpreted by Air Force toxicologists to determine the hazards these effluents pose to human health.

MATERIALS AND METHODS

Animals

New Zealand White rabbits, weighing between 2 and 3 kg, will be used in the dermal toxicity, eye irritation, and skin irritation studies. Male and female Fischer-344 rats weighing 150 to 250 g (males) and 125 to 250 g (females) will be used to evaluate oral toxicity of the test compounds. Male, albino, Hartley-strain guinea pigs, weighing between 200 and 250 g upon receipt, will be used in the sensitization tests. Quality control determinations will be made on the animals during a two-week quarantine period to determine if they are in acceptable health. The rabbits and guinea pigs will be housed individually, the guinea pigs in plastic cages with wood chip bedding and the rabbits in wire-bottom stainless steel cages. The rats will be housed three to four to a plastic cage with wood chip bedding. Water and feed will be available *ad libitum*. All animals will be maintained on a 12-h interval light/dark cycle.

All animals living at the termination of the observation period of the acute tests will be sacrificed for gross pathological examination. Any animal that dies following treatment will also be subjected to gross necropsy. No tissues will be taken.

Test Materials

The test materials will be provided and identified by the Air Force. Chemical analysis of individual samples will be performed by Air Force personnel. Upon receipt at the Toxic Hazards Research Unit, the test materials will be maintained at 2-4°C and continuously mixed using a magnetic stir plate prior to testing. The NSI-ES Chemistry Section will retain archive samples of all test materials; these archived samples will be stored in the chemical storage cabinets in Building 429 at ambient temperature.

Oral Toxicity

Approximately 30 male 7- to 10-week-old Fischer-344 rats weighing 150 to 250 g and 30 females weighing 125 to 250 g will be divided into four treatment groups (Table 3.4.2-1). The rats will be fasted for at least 16 h before the administration of the dose by oral gavage. Just prior to dosing, each rat will be weighed to determine dose volume. A control group will be maintained for body weight measurements only. Dilutions (if required) will be made in distilled water or other appropriate vehicle.

TABLE 3.4.2-1. TREATMENT GROUPS FOR ORAL TOXICITY TESTING

Group	Dose, g/kg	No. Animals	Assessment
I – Limit Test	5.0[a]	5 male 5 female	Body weight, toxic signs, mortality
II – Control	5.0 (distilled water or vehicles)	5 male 5 female	
III – LD_{50}	[b]	10 male 10 female	Body weight, toxic signs, mortality
IV – Control	(distilled water or vehicle)	10 male 10 female	

[a] In the case of liquid slurry samples, a volume of undiluted material equivalent to 5 g will be administered to each rat.

[b] If deaths occur at the limit test, at least three dose levels will be used and spaced appropriately to produce a range of toxic effects and mortality rates sufficient to permit the determination of an LD_{50}.

Surviving rats will be weighed at 1, 2. 3, 7, 10, and 14 days. Toxic signs will be recorded on symptomatology forms, and gross pathology will be performed on all animals.

If deaths occur at the limit test, additional dose levels will be tested. To reduce the numbers of animals required, geometrically spaced doses will be administered and the LD_{50} calculated using the moving average method of Weil (1952). A repeated measures test will be used to compare body weights against controls (Barcikowski, 1983).

Dermal Toxicity

Five male and five female New Zealand White rabbits, weighing between 2 and 3 kg, will be administered a maximum dose volume of 2.0 g test material/kg body weight. The undiluted test material will be applied to the clipped back of the rabbit and spread evenly to both sides. The test material will be kept in place by 8-ply gauze patches, and a clear plastic wrap will then be applied over the entire midsection. The patch will be held in place with Vetrap® and Elastoplast® tape. After 24 h, the tape, plastic wrap, and gauze will be removed, and residual test material will be wiped from the animal. Table 3.4.2-2 shows the experimental design.

TABLE 3.4.2-2. EXPERIMENTAL DESIGN FOR TOXICITY TESTING

Group	Dose, g/kg	No. Animals	Assessment
I – Limit Test	2.0	5 male 5 female	Body weight, toxic signs, mortality
II – LD_{50}	[a]		

[a] If deaths occur at the limit test, at least three dose levels will be used and spaced appropriately to produce a range of toxic effects and mortality rates sufficient to permit the determination of an LD_{50} (an additional 20 rabbits of each sex would be needed for this phase).

Body weights will be recorded at the time of dosing and on days 1, 2, 4, 7, 10, and 14 posttreatment. Symptoms will be recorded on symptomatology forms. Gross pathology will be done on all animals; no histopathology will be required.

If deaths occur at the limit test, additional dose levels will be tested. To reduce the numbers of animals required, geometrically spaced doses will be administered and the LD_{50} calculated using the moving average method of Weil (1952).

Eye Irritation

Nine New Zealand White rabbits of either sex, weighing between 2 and 3 kg, will be used for this study. Prior to testing, the pH of the test materials will be determined. If the pH is less than 2.0 or greater than 11.5, testing for eye irritation will not be done because of predictable corrosive properties.

The eyes of the test rabbits will be examined with fluorescein stain prior to use to ensure absence of lesions or injury. A topical anesthetic will be instilled in the eyes of all rabbits, treated and control groups, approximately 2 min prior to application of the test substance. One-tenth milliliter of the undiluted material will be applied to one eye of each of the nine rabbits. The opposite eye will remain untreated to serve as a control. The treated eyes of six rabbits will remain

unwashed, while the treated eyes of remaining three rabbits will be flushed for one min with lukewarm water starting 30 s after instillation. The experimental design is shown in Table 3.4.2-3.

TABLE 3.4.2-3. EXPERIMENTAL DESIGN FOR EYE IRRITATION TESTING

Group	Dose, g/kg	No. Animals	Assessment
I	0.1 ml	9	Irritation
II-LD_{50}	[a]		

[a] If deaths occur at the limit test, at least three dose levels will be used and spaced appropriately to produce a range of toxic effects and mortality rates sufficient to permit the determination of an LD_{50} (an additional 20 rabbits of each sex would be needed for this phase).

The rabbits will be examined for gross signs of eye irritation at 1, 24, 48, and 72 h following application. Irritative effects will be second according to the method of Draize (1959). No gross pathology or histopathology is required.

Skin Irritation

Prior to testing, the pH of the test material will be determined. If the pH is less than 2.0 or greater than 11.5, testing for skin irritation will not be done because of predictable corrosive properties.

Six New Zealand White rabbits from the eye irritation study will be utilized. The rabbits will be of either sex and will weigh between 2 and 3 kg. One-half milliliter of the undiluted test agent will be applied to the designated patch area. The treated area will be covered by a 2.5-cm square of surgical gauze, which will be held in place with strips of Elastoplast® tape. The entire area will be covered with dental dam and secured with Vetrap® and Elastoplast® tape. The patches will remain in place for 4 h, at which time all wrappings will be removed and residual test agent wiped from the animals.

Rabbits will be examined for signs of skin irritation at 4, 24, 48, and 72 h. Irritative effects will be scored according to the method of Draize (1959).

Sensitization

Test I – Primary Irritation. One-tenth of a milliliter of test material will be applied topically to the left flank of three guinea pigs. If the test material is irritating to the guinea pigs' skin, the test material will be diluted and the guinea pigs redosed at a different site. One site will be used for the vehicle and another for the diluted test material.

Test II – Sensitization. (A) Baseline Irritation Response: Prior to the start of the study, each guinea pig will be dosed with 0.1 mL of the material on the left flank to evaluate baseline irritation response. Animals showing irritative response at this time will not be included in the test.

(B) Sensitization Reactions: Test solutions will be applied to a clipped and chemically depilated area on the back of each animal directly above its forelegs. The area will be covered with a 1.5- x 1.5-cm gauze square and covered with dental dam. The patch will be held in place with adhesive tape. These applications will be made on Mondays, Wednesdays, and Fridays until a total of four sensitizing doses have been applied. Along with the third sensitizing dose, 0.2 mL of a 50% aqueous dilution of Freund's adjuvant (Bacto Adjuvant Complete, Freund, Difco Laboratories, Detroit, MI) per animal will be injected intradermally, using two to three sites next to the test site.

(C) Challenge Dose: Following the fourth sensitizing dose, the animals will be rested for two weeks. Both flanks will then be clipped; one flank will be challenged with the test solution and the other flank with the vehicle (if any). The challenge applications will not be occluded.

Examination for signs of a sensitization reaction will be made at 24 and 48 h following the challenge dose. Any animal eliciting a score of 2 or more at the test solution challenge site at the 48-h scoring will be rated a positive responder. Grading will be as follows:

Erythema	Edema
0 – None	0 – None
1 – Very Slight pink	1 – Very slight
2 – Slight pink	2 – Slight
3 – Moderate red	3 – Moderate
4 – Very red	4 – Marked

In scoring this test, the important statistic is frequency of the reaction. The following method is used to classify test materials as to sensitization potential.

Sensitization Rate	Grade
10	Weak
20-30	Mild
40-60	Moderate
70-80	Strong
90-100	Extreme

Table 3.4.2-4 shows the experimental design.

TABLE 3.4.2-4. EXPERIMENTAL DESIGN FOR THE SENSITATION TESTS

Test	Dose[a]	No. Animals	Assessment
I	0.1 mL	3	Identification of a nonirritating concentration of test material to be used to determine sensitization
IIA	0.1 mL	10[b]	Baseline irritation response
B	0.1 mL	10[b]	Sensitization reactions
C	0.1 mL	10[b]	Challenge dose to determine sensitization reactions

[a] The dose volume will remain constant; however, the test material may require dilution, depending upon the irritation response.
[a] Represents the same groups of 10 animals.

Repeated Oral Toxicity (14-day)

Male and female Fischer-344 rats, weighing 200 to 300 g and 150 to 250 g, respectively, will be used in this study. The animals will be dosed daily, excluding weekends (10 dosing days over a 2-week period beginning on a Wednesday). The dosage volumes will be calculated from individual body weights and adjusted weekly. The test material will be administered by gavage at the beginning of each day prior to 0930 hours. The control group will receive an equal volume of distilled water. Food will be provided after gavage and removed at 1615 hours each day. Water will be provided *ad libitum*. Test and control groups will be sacrificed on the day following the 10th dose. Table 3.4.2-5 shows the experimental design.

TABLE 3.4.2-5. EXPERIMENTAL DESIGN FOR THE REPEATED ORAL TOXICITY TESTS

Group	Dose	No. Animals	Assessment
I	5.0[a] (test material)	10 male 10 female	Clinical signs, body weight, hematology, clinical chemistry, pathology
II	5.0 (distilled water)	10 male 10 female	same as above same as above

[a] In case of liquid or slurry samples, a volume of undiluted material equivalent to 5 g will be administered.

The rats will be weighed on days 0, 7, and 14. Whole blood assays (from 5 rats of each sex per group) and blood serum chemistries will be performed by Air Force personnel, using heparinized whole blood collected from the posterior vena cava at sacrifice.

Whole Blood Assays	Serum Chemistries
White blood cell count	Chloride
Red blood cell count	Serum glutamic-oxalacetic transaminase
Hemoglobin	Blood urea nitrogen
Mean corpuscular volume	Creatinine
Hematocrit	Alkaline phosphatase
Mean corpuscular hemoglobin	Calcium
Differential leucocyte count	Sodium
Mean corpuscular hemoglobin concentration	Phosphorus
	Potassium
	Total bilirubin
	Total protein
	Serum glutamic-pyruvic transaminase

On the day of sacrifice the following tissue weights will be recorded: adrenals, brain, heart, kidneys, liver, lungs, ovaries, spleen, and testes.

A gross pathologic examination will be performed on each rat and the following tissues taken for histopathologic examination.

gross lesions	ileum	gonads
kidneys	urinary bladder	uterus
trachea	mandibular lymph nodes	esophagus
heart	mesenteric lymph nodes	stomach
liver	sciatic nerve	cecum
spleen	lungs	colon
duodenum	adrenals	rectum
jejunum	pancreas	sternum

The following statistical analyses will be performed: body weight – repeated multivariate analysis of variance with Scheffe pair-wise comparisons (Barcikowski, 1983); hematology – a two-factorial analysis of variance with multiple comparisons (Barcikowski, 1983); clinical chemistry – a two-factorial analysis of variance with multiple comparisons (Barcikowski, 1983); and histopathology – one of the following nonparametric tests: Fisher's Exact Test or, if not valid, Yates' Corrected Chi-Square (Zar, 1974).

Repeated Dermal Toxicity (14-Day)

Male and female (10 each per test group) New Zealand White rabbits, weighing between 2 and 3 kg, will be dosed daily, excluding weekends (10 dosing days over a 2-week period beginning on a Wednesday). The dose volumes will be calculated from individual body weights and adjusted weekly. The test material will be held in contact with the skin for 6 h per day. The control group will receive an equivalent dose of distilled water. Test and control groups will be sacrificed on the day following the 10th dose. Table 3.4.2-6 shows the experimental design.

TABLE 3.4.2-6. EXPERIMENTAL DESIGN FOR THE REPEATED DERMAL TOXICITY TESTS

Group	Dose	No. Animals	Assessment
I	2	10 male 10 female	Clinical signs, body weight, hematology, clinical chemistry, pathology
II	2 (distilled water)	10 male 10 female	same as above same as above

A clinical examination will be made daily and any signs of toxicity will be recorded. The rabbits will be weighed at 0, 7, and 14 days posttreatment. For blood assays to be performed by Air Force personnel, blood will be taken prior to initiation of dosing and at sacrifice from five animals of each sex per group. The blood will be sampled via the marginal ear vein and collected in heparinized tubes. The following whole blood assays and serum chemistries will be performed.

Whole Blood Assays	Serum Chemistries
White blood cell count	Creatinine
Hematocrit	Chloride
Hemoglobin	Calcium
Red blood cell count	Phosphorus
Total and differential blood cell count	Total protein
	Alkaline phosphatase
	Blood urea nitrogen
	Serum glutamic-pyruvic transaminase
	Serum glutamic-oxalacetic transaminase

The following wet tissue organ weights will be measured at sacrifice: adrenals, brain, heart, kidneys, liver, lungs, ovaries, spleen, and testes.

A gross pathologic examination will be performed on each rabbit; any gross lesions and the following tissues will be taken for histopathologic examination.

normal and treated skin	gall bladder
lungs	kidneys
trachea	adrenals
heart	pancreas
liver	gonads
spleen	uterus
duodenum	esophagus
jejunum	stomach
ileum	cecum
urinary bladder	colon
mandibular lymph nodes	rectum
mesenteric lymph nodes	sternum
sciatic nerve	

The following statistical analyses will be performed: body weight – repeated multivariate analysis of variance with Scheffe pair-wise comparisons (Barcikowski, 1983); hematology – a two-factorial analysis of variance with multivariate comparisons (Barcikowski, 1983); clinical chemistry – a two-factorial analysis of variance with multivariate comparisons (Barcikowski, 1983); organ weights – a two-factorial analysis of variance with multivariate comparisons (Barcikowski, 1983); and histopathology – one of the following nonparametric tests: Fisher's Exact Test or, if not valid, Yates' Corrected Chi-Square (Zar, 1974).

REFERENCES

Barcikowski, R.S. 1983. *Computer Packages and Research Design Vol 1, BMDP.* Lanham, MD: University Press of America.

Draize, J.H. 1959. *Dermal Toxicity, Appraisal of the Safety of Chemicals in Food, Drugs, and Cosmetics*, pp. 46-49. The Staff of the Division of Pharmacology of the Federal Food and Drug Administration, eds. Austin, TX: The Editorial Committee of the Associates of Food and Drug Officials of the United States.

Weil, C. S. 1952. Tables for convenient calculation of median-effective dose (LD_{50} or ED_{50}) and instructions in their use. *Biometrics* 8:249-263.

Zar, J.H. 1974. *Biostatistical Analysis.* Englewood Clifts, NJ: Prentice Hall.

3.5 CHEMICAL DEFENSE TOXICOLOGY

3.5.1 Evaluation of the Acute Toxicity of Diisopropylaminoethanol

E. R. Kinkead and D. L. Pollard

INTRODUCTION

Because of the possibility of exposure of military and civilian personnel to O-ethyl-O'-(2-diiosopropylaminoethyl)methylphosphonite (QL) manufacturing by-products, the U.S. Army has a strong interest in characterizing the acute toxicity of some of its precursors, intermediates, and by-products. The unstable character of QL has been previously demonstrated (Vernot *et al.*, 1984) and the breakdown products are thought to directly affect the acute toxicity attributed to QL. One of these compounds of interest is diisopropylaminoethanol (KB).

This study was designed to characterize the toxic effects of acute exposure of rats, mice, and rabbits to KB. The compound was tested in the same manner as was used for the acute toxicity testing of QL (Vernot *et al.*, 1984). The data obtained from this experiment should permit objective assignment of occupational exposure limits for personnel working with KB.

MATERIALS AND METHODS

Test Material

The KB used in this study was furnished by the U.S. Army. KB is an alkyl alkanol amine that has a moderate amine odor and amber color. Table 3.5.1-1 lists the known physical characteristics of KB.

TABLE 3.5.1-1. PHYSICAL CHARACTERISTICS OF KB

Chemical Formula	$(C_3H_7)_2\ NC_2H_4OH$
Mol. Wt.	145.28
pH	9.9
Density	0.870 g/mL
Boiling point	188-192°C
Water solubility	1.3% at 20°C
Flash point	67°C

Quality Control

The purity of the KB was tested using capillary gas chromatography. A Varian 3700 gas chromatograph (GC) equipped with a flame ionization detector and a 50-m fused silica SE-54 capillary column was used. A Hewlett-Packard 3388 computing integrator was used to measure the

peak areas and record the chromatograms. The purity of the KB remained greater than 99% for the duration of the study.

Animals

Male and female Sprague-Dawley rats, Fischer-344 rats, and Swiss-derived mice were used in these studies. The animals were fed Purina Laboratory chow and housed in an animal facility providing a 12-h, light-dark cycle with controlled heat and humidity.

Oral Toxicity

Male and female Sprague-Dawley rats and male and female CD-1 mice were used for the oral testing of KB. Geometrically spaced doses were administered to determine the LD_{50} values for 10 animals of each sex at each level. The LD_{50} values with 95% confidence limits were calculated using the probit method of Finney (1971).

All compounds were dissolved or suspended in corn oil, and the suspensions kept in a turbulent state using a magnetic stirring platform during the dosing procedure. Glass syringes with oral dosing needles were used to administer the compounds to rats and mice that had been fasted for at least 16 h prior to administration of the oral dose. A dose volume of 0.01 mL/g body weight was administered to animals that had been individually weighed at the time of dosing.

Skin Irritation

A patch-test method was utilized to determine the degree of primary skin irritation of intact skin of six albino rabbits. The hair on rabbits' back and flanks was clipped as closely as possible 24 h prior to exposure to allow the skin to recover from any abrasion resulting from the clipping. Undiluted test material (0.5 mL) was applied to the designated patch area and covered by surgical gauze. The patch was held in place with strips of surgical adhesive tape. The entire area was covered with polyethylene plastic wrap and secured with Elastoplast® tape. After 4 h the wrap and patch were carefully removed, excess material was wiped from the skin, and the test area was evaluated for irritation using the Draize (1959) table as a reference standard. Irritation evaluations were also made at 72 h after treatment. A primary index rating was calculated using the following formula:

$$\text{Primary Irritation Index} = \frac{\text{Total Reaction Score}}{(\text{No. of Rabbits})\,(\text{Test Sites/Rabbits})\,(\text{Times Scored})}$$

The primary index score was interpreted using the the NIOSH skin test ratings (Campbell *et al.*, 1975).

Aerosol Generation System

KB aerosol was produced using Solosphere Nebulizers (American Pharmaseal, Cat. #H2500/H2501). Two nebulizers were required to produce the highest concentration of test agent (2,592 mg/m^3). Figure 3.5.1-1 shows a schematic of the introduction system. The aerosol was generated into a 40-L/min air stream connected to the chamber's input air system. Chamber airflows, adjusted to assist in the control of the concentration of KB, varied between 0.6 and 1.0 chamber volumes per min. KB was supplied to the nebulizer(s) from a 2-L polyethylene reservoir using a metering pump. The KB concentration during exposure was maintained by adjusting the input airflow to the nebulizer.

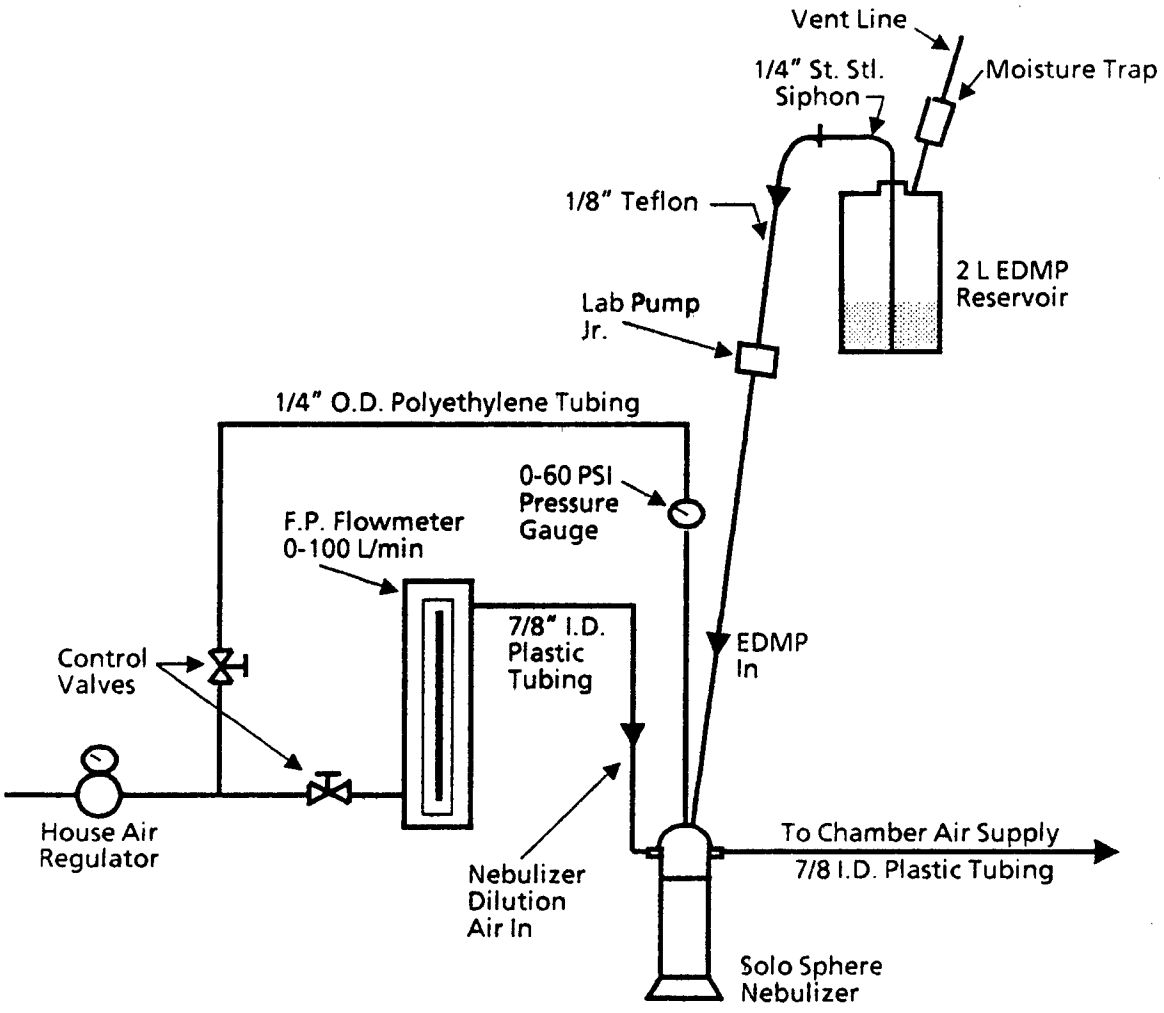


Figure 3.5.1-1. KB Aerosol Generation System.

Gas Chromatographic Analysis of Chamber Atmosphere

To determine if any decomposition of the KB was occurring during the exposure, samples of the chamber atmosphere were analyzed using a GC. Sampling was accomplished by pulling 10 L of chamber air through a midget impinger containing 20 mL of diethyl ether. Two microliters of the trapping solution were injected into a Model 3700 Varian GC equipped with a 50-m SE-54 fused silica capillary column. The column was temperature programmed from 100° to 170°C at 4°/min. The output was collected on a Hewlett-Packard 3388 (Palo Alto, CA) computing integrator.

Analysis of Chamber Atmosphere for Residual Aerosol

To assess the completeness of KB volatilization from the generated aerosol the concentration of particles in the chamber atmosphere was analyzed during each exposure. These aerosol measurements were made using either a cascade impactor (Andersen, Atlanta, GA) or an optical particle counter (Royco, Model 227, Menlo Park, CA). Baseline data were recorded prior to aerosol generation and generally three measurements were made during each 6-h exposure.

Animal Exposures

Groups of 10 male and 10 female Fischer-344 rats and 10 male and 10 female CD-1 mice, all 9 to 11 weeks old, were used for the determination of the 6-h LC_{50} values. Control groups were maintained in animal rooms for comparative purposes.

Ten animals per sex of each species were exposed at each concentration and an LC_{50} with 95% confidence limits was calculated using the probit method of Finney (1971). The animals were observed frequently during the exposure and twice daily during the 14-day holding period. Visible signs of toxicity were recorded, and all animals were weighed prior to exposure and at 1, 2, 4, 7, and 14 days postexposure.

A gross histologic examination was performed on any animal that died during the exposure or the 14-day observation period. All surviving animals were observed for 14 days or until signs of reversible toxicity subsided, at which time they were sacrificed by halothane inhalation. Gross examination of lung, liver, kidneys, heart, stomach, small and large intestine, spleen, and brain was performed on each animal.

Oral Toxicity

Ten male and ten female Sprague-Dawley rats and 10 male and 10 female CD-1 mice received oral doses of KB in corn oil. Oral toxicity to both sexes of rats and mice was essentially the same, falling between a low LD_{50} value of 0.77 g/kg for male mice and a high LD_{50} value of 1.08 g/kg for female mice.

Oral doses of KB produced tremors and convulsions in both rats and mice prior to death, which generally occurred within 24 h postdosing. Porphyrin exudate was observed around eyes and noses of the survivors for several days following the oral dose.

Skin Irritation

A summary of the skin irritation effects of KB has been provided in Table 3.5.1-2. This compound proved to be a severe irritant and a corrosive as indicated by the necrosis it produced in all six test rabbits. The primary index score of 9.0 classifies KB as too irritating for human skin contact.

TABLE 3.5.1-2. SUMMARY OF RABBIT SKIN IRRITATION EFFECTS AFTER 4-HOUR CONTACT WITH KB

4 Hours	24 Hours	72 Hours
Well-defined (5) to severe (1) erythema. Very slight (4) to slight (2) edema. No necrosis (6)	Well-defined erythema (6). None (1), Very slight (2) to slight (3) edema. Slight necrosis (6)	Moderate to severe erythema (6). Slight edema (6). Moderate necrosis (6)

Primary Index Score = 9.0
Classification: severe irritant
( ) Number of rabbits showing symptoms

The effect of KB contact was evident on the skin of rabbits through four weeks following treatment. After one-week posttreatment, the contact areas showed signs of corrosion with sloughing of the dead tissue. However, at eight weeks posttreatment, the necrotic areas were completely healed. Because KB proved to be a severe irritant and a corrosive in the skin irritation test, the eye irritation and dermal LD_{50} studies were not conducted.

Inhalation Toxicity

The results of the GC analysis of chamber atmospheres showed that decomposition of KB did not occur during the 6-h exposures. All of the KB breakdown products or impurities, if present, were below the level of detection. Aerosol concentrations were less than 8 mg/m^3 (less than 0.32% of the atmospheric concentration) in all exposures.

A summary of the 6-h inhalation toxicity effects of KB on male and female rats and mice is provided in Tables 3.5.1-3 and 3.5.1-4, respectively. Inhalation of KB vapors resulted in immediate eye and nose irritation followed by fine tremors, and labored breathing and gasping, with convulsions usually preceding death. Deaths generally occurred within 24 h postexposure. The calculated inhalation LC_{50} values for male and female rats were 2,041 mg/m^3 and 1,965 mg/m^3, respectively. No appreciable difference in toxic response was noted between male and female rats exposed to KB.

KB appeared to be somewhat more toxic to female mice than male mice upon comparison of the relative LC_{50} values (Table 3.5.1-4). The apparent increased toxicity resulted from the reversal in mortality that occurred in a group of female mice exposed to 1,676 mg/m^3 KB. This reversal also precluded the calculation of confidence limits for the female mouse LC_{50}.

TABLE 3.5.1-3. SUMMARY OF 6-HOUR INHALATION TOXICITY RESULTS OF KB ON FISCHER-344 RATS (N = 10)

Concentration, mg/m^3	Mortality Ratio	
	Males	Females
2,592	10/10	10/10
2,105	6/10	8/10
1,836	2/10	2/10
1,766	0/10	1/10

Male rat LC_{50} (95% c.l.) = 2041 (1942-2223) mg/m^3
Female rat LC_{50} (95% c.l.) = 1965 (1877-2108) mg/m^3

TABLE 3.5.1-4. SUMMARY OF 6-HOUR INHALATION TOXICITY RESULTS OF KB ON CD-1 MICE (N = 10)

Concentration, mg/m^3	Mortality Ratio	
	Males	Females
2,169	8/10	10/10
1,989	5/10	9/10
1,727	----	7/10
1,676	0/10	10/10
1,584	----	2/10
1,568	0/10	0./10

Male mouse LC_{50} (95% c.l.) = 2011 (1869-2119) mg/m^3
Female mouse LC_{50} = 1661 mg/m^3 (95% c.l. unobtainable)

DISCUSSION

KB is corrosive to the skin, and although KB was not tested for ocular irritation, it must be assumed to be damaging to the eyes. The corrosiveness of this compound would prove hazardous in the event of body contact. The compound must be considered toxic through oral ingestion with LD_{50} values ranging between 0.77 g/kg and 1.08 g/kg. However, the observed oral toxicity was not much different than the oral LD_{50} values reported for QL; these values ranged from 0.71 g/kg to 2.75 g/kg (Vernot *et al.*, 1984).

The experimental inhalation LC_{50} of KB (approximately 2000 mg/m^3) was similar to the 6-h LC_{50} of QL in male rats (2,520 mg/m^3) and male and female mice (2300 to 3500 mg/m^3; Vernot *et al.*, 1984). The increased sensitivity noted in female rats exposed to QL inhalation was not apparent

following inhalation of KB and did not appear to be directly related to the amount of KB present in the QL exposures.

REFERENCES

Campbell, K.I., E.L. George, L.L. Hall, and Stara, J.F. 1975. Dermal irritancy of metal compounds, *Arch Environ. Health* 30:168-170.

Draize, J.H. 1959. Dermal toxicity. In: The Staff of the Division of Pharmacology of the Federal Food and Drug Administration, ***Appraisal of the Safety of Chemicals in Food, Drugs and Cosmetics***, pgs. 46-49. Austin, TX: The Editorial Committee of the Associates of Food and Drug Officials of the United States.

Finney, D.J. 1971. *Probit Analysis*, 2nd Edition, p. 131. Cambridge, England: Cambridge University Press.

Vernot, E.H., J.D. MacEwen, E.R. Kinkead, J.R. Horton, J.D. Collins, W.J. Bashe, C.C. Haun, A.K. Roychowdhury, R. A. Salomon, and E. J. Olajos. 1984. *Studies on the Acute Toxicity of O-ethyl-O'-(2-diisopropylaminoethyl)methylphosphonite (EDMP)*. CRDC-CR-84116. Aberdeen Proving Ground, MD: U.S. Army Armament, Munitions and Chemical Command.

3.5.2 Evaluation of the Acute Toxicity of Triethylphosphite

E. R. Kinkead and D. L. Pollard

Because of the possibility of exposure of military and civilian personnel to O-Ethyl-O'(diisopropylaminoethyl)methylphosphonite (QL) manufacturing by-products, the Army has a strong interest in characterizing the acute toxicity of some of its precursors, intermediates, and by-products. The unstable character of EDMP has been previously demonstrated (Vernot *et al.*, 1984), and the breakdown products are thought to directly affect the acute toxicity attributed to EDMP. One of the compounds of interest is triethylphosphite (TEP).

This study was designed to characterize the toxic effects of acute exposure of rats, mice, and rabbits to TEP. The compound was tested in the same manner as was used for the acute toxicity testing of diisopropylaminoethanol (KB). The data obtained from this experiment should permit objective assignment of sound occupational exposure limits for personnel working with TEP.

MATERIALS AND METHODS

Test Material

The TEP used in this study was purchased from the Stauffer Chemical Company, Westport, CT. TEP is an organic phosphorus compound with a very strong odor and a colorless appearance. TEP hydrolyzes in the presence of water or moist air to form diethylphosphite and ethanol. It can also be oxidized to triethylphosphate after prolonged exposure to those conditions. Table 3.5.2-1 lists selected known physical characteristics of TEP.

TABLE 3.5.2-1. PHYSICAL CHARACTERISTICS OF TEP

Chemical Formula	$(C_2H_5O)_3P$
Mol. Wt.	166.16
pH	5.9
Density	0.955 g/mL
Boiling point	157.9°C
Water solubility	Insoluble
Flash point	54.4°C

Quality Control

A Varian 3700 gas chromatograph (GC) equipped with a flame ionization detector and a 50-m fused silica SE-54 capillary column was used to determine purity of the TEP. A Hewlett-Packard 3388

computing integrator was used to measure the peak areas and record the chromatograms. The purity of the TEP was found to be between 95 and 97%.

Oral Toxicity

All compounds were dissolved or suspended in corn oil, and the suspensions kept in a turbulent state using a magnetic stirring platform. Glass syringes with oral dosing needles were used to administer the compounds to the rats and mice. All animals were fasted for at least 16 h prior to administration of the oral dose. The animals were weighed individually at the time of dosing, and the dose volume administered was 0.1 mL/gm body weight. Male and female F-344 rats and male and female CD-1 mice were used. Geometrically spaced doses were administered to determine the LD_{50} in ten animals of each sex at each dose level. The LD_{50} values with 95% confidence limits were calculated using the probit method of Finney (1971).

Skin Irritation

A patch test method was utilized to determine the degree of primary skin irritation on the intact skin of six albino rabbits. The hair on the rabbits' back and flanks was clipped as closely as possible 24 h prior to exposure to allow the skin to recover from any abrasion resulting from the clipping. Undiluted test material (0.5 mL) was applied to the designated patch area and covered by surgical gauze. The gauze was held in place with strips of surgical adhesive tape. The entire area was covered with polyethylene plastic wrap and secured with Elastoplast® tape. After 4 h the wrap and patch were carefully removed, and the test area was evaluated for irritation using the Draize (1959) table as a reference standard. Readings were also made at 72 h after treatment. A primary index rating was calculated using the following formula:

$$\text{Primary Irritation Index} = \frac{\text{Total Reaction Score}}{(\text{No. of Rabbits})\,\text{Test Sites/Rabbits})\,(\text{Times Scored})}$$

The primary index score was interpreted using the NIOSH skin test ratings (Campbell *et al.*, 1975).

Eye Irritation

The test compound (0.1 mL) was applied to one eye of each of nine albino rabbits. The opposite eye was untreated and served as a control. The eyes were examined with fluorescein stain prior to use to ensure absence of lesions or injury. A topical anesthetic was instilled in the eyes of all rabbits, treated and control, approximately 2 min prior to application of the test substance. The treated eye of 6 rabbits remained unwashed, while the treated eye of the other 3 rabbits was flushed for 1 min with lukewarm water starting no sooner than 20-30 s after instillation of the test compound. The rabbits' eyes were examined for gross signs of eye irritation 1, 2, 3, 4, and 7 days

following application. If an injury occurred, the effects were scored three times a week until the lesion subsided or was deemed irreversible. The irritative effects were scored according to the method of Draize (1959).

Dermal Toxicity

Male and female albino New Zealand rabbits, weighing between 2 and 3 kg, were used. The rabbits' backs and sides about halfway down to the abdomen were clipped from the saddle area of the shoulders to the top of the rear leg area.

The animals were individually weighed prior to dosing to determine the proper dose volume. The dose was applied undiluted to the backs of the rabbits and divided as equally as possible between the two sides. The test material was kept in place by 8-ply gauze patches. A patch of clear plastic wrap was then applied over the entire clipped back area and Elastoplast® tape was used to wrap the entire midsection of the rabbit. Specially designed restraining harnesses were fitted to the rabbits at the time of dosing and kept in place during the entire treatment period. These harnesses prevented the rabbits from excessive movement and from chewing on the taped area, while allowing them access to food and water during the dosing period.

All doses were kept in contact with the rabbits' skin for 24 h. The animals were observed frequently on the day of dosing and twice daily thereafter, and all symptoms were recorded. Body weights were obtained at the time of dosing and on days 1, 2, 4, 7, 10, and 14 posttreatment. The rabbits were observed for mortality or other signs of toxicity during the 14 days immediately following exposure. Any deaths that occurred during this period were included in the final mortality results.

Testing was initiated by dosing five animals of each sex with 2 mL of test material/kg of body weight. If there was no appreciable mortality at this level, no additional dose levels were administered. If mortality was observed, testing was continued with five animals of each sex per dose level. LD_{50} values were calculated using the probit method of Finney (1971).

Aerosol Generation System

TEP aerosol was introduced into the chamber by using either two or three Solosphere nebulizers (American Pharmaseal, Cat. #H2500/H2501) depending on the concentration required. Figure 3.5.2-1 shows a schematic of the introduction system. Compressed air was metered to the nebulizers to cause aerosolization. A 100 L/min supply of compressed air was used to carry the TEP aerosol to the chamber air input line. Additional TEP was supplied to the nebulizer(s) from a 2-L polyethylene bottle using a Lab Pump Jr. (Fluid Metering Inc., Oyster Bay, NY). During the exposures, TEP concentration was controlled by regulating the input air pressure. The chamber air flow was also used to help control the concentration.

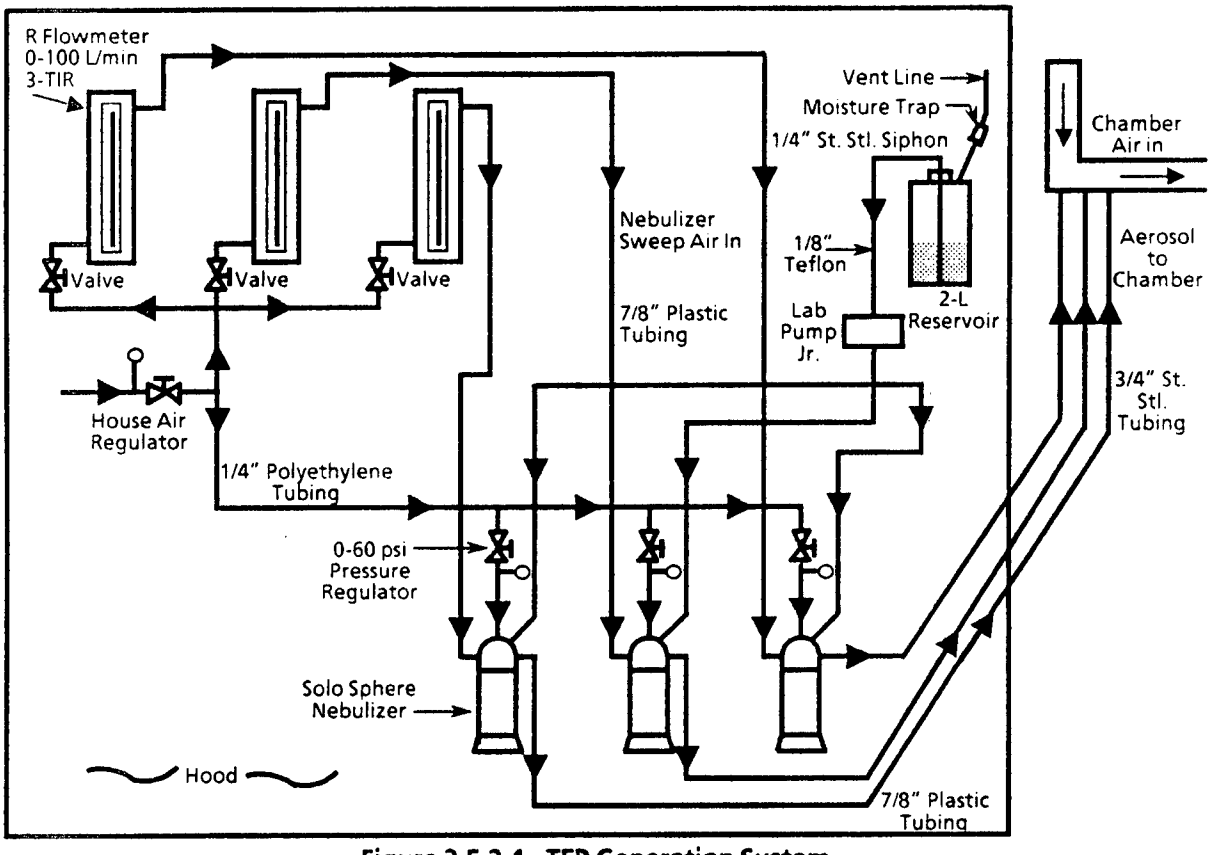


Figure 3.5.2-1. TEP Generation System.

Gas Chromatographic Analysis of Chamber Atmosphere

To determine chamber concentration as well as monitor any decomposition of the TEP, samples of the chamber atmosphere were analyzed by gas chromatography. Chamber atmospheres were sampled by pulling 10 L of air out of the chamber through a midget impinger containing 20 mL of diethyl ether. The trapping solution (2.0 µL) was then injected onto a 50-m SE-54 fused silica capillary column in a Varian 3700 GC. The column oven was temperature programmed from 100° to 230°C at 4°/min.

Analysis of Chamber Atmosphere

The concentration of TEP in the chamber was monitored using a Miran 1A infrared analyzer equipped with a zinc selenide liquid flowcell (Wilks Foxboro 0.5-mm pathlength Model #420-2-A-3-1-C). Figure 3.5.2-2 is a schematic of the analytical system, which employed glass spiral absorber towers. One tower was positioned inside the chamber, while the other was used for the input baseline air sample. Both towers were packed with glass beads. Samples of baseline air or chamber atmosphere were drawn into the top of the tower at a rate of 2.5 L/min. A solvent, 4-methylcyclohexanol (MCHOH), was pumped into the top of the tower at 1.0 mL/min using a polystaltic pump. As the sample air and the MCHOH traveled down the tower and mixed, the TEP was absorbed by the MCHOH. Another polystaltic pump pulled the MCHOH (from the bottom of the absorber towers) containing the absorbed TEP into the Miran analyzer. Light absorption at 11.0 µ was used to determine the concentration of TEP in the MCHOH.

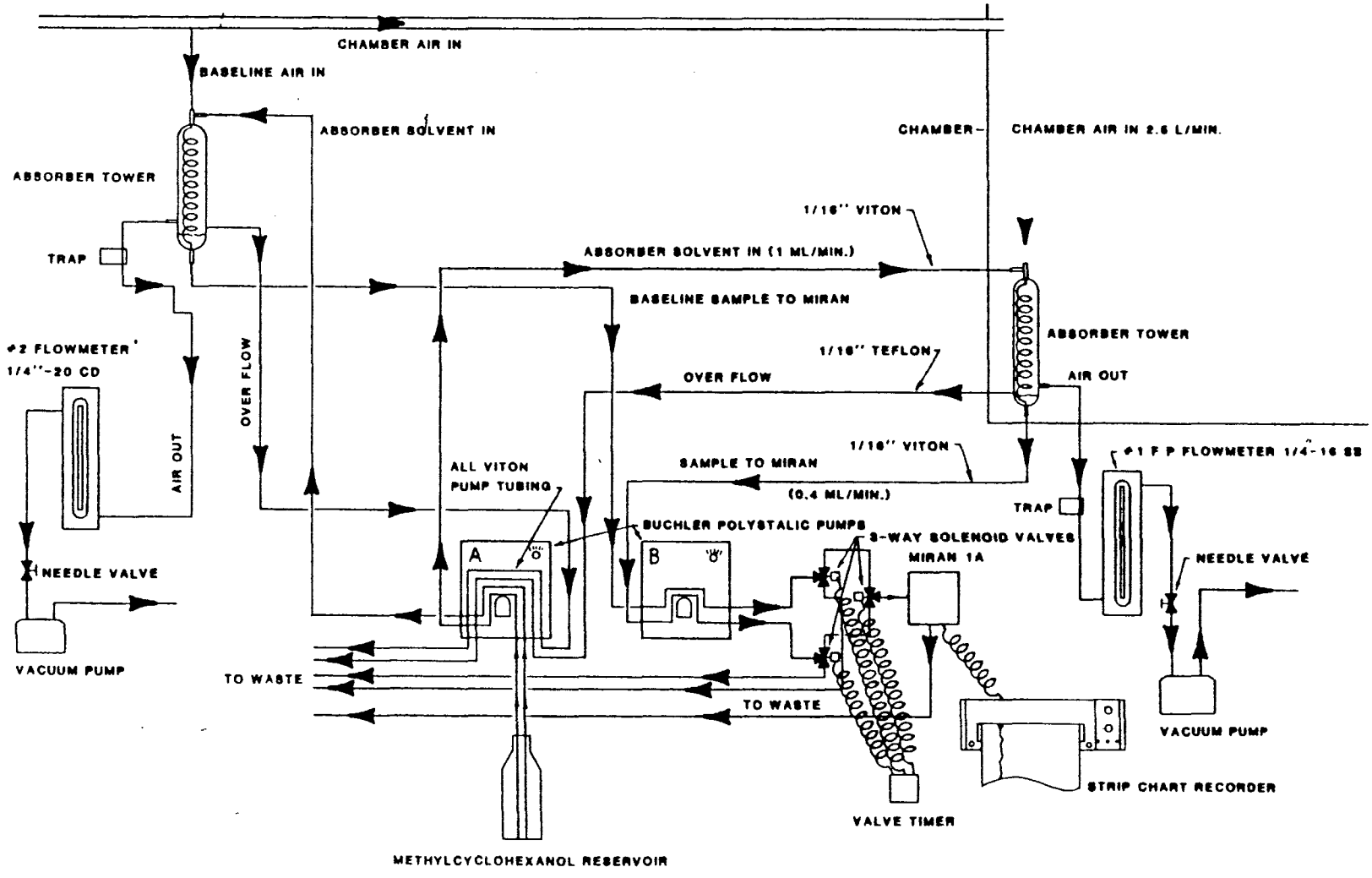


Figure 3.5.2- 2. Miran Analytical System.

Animals

Male and female Fischer-344 rats and Swiss-derived mice were used in the inhalation exposure studies. The animals were fed Purina Laboratory Chow and housed in an animal facility providing a 12-h light-dark cycle with controlled heat and humidity.

Exposure Regimen

Groups of 10 male and 10 female Fischer-344 rats and 10 male and 10 female CD-1 mice, all 9 to 11 weeks old, were used for the determination of a 6-h LC_{50}. Control groups were maintained in animal rooms for comparative purposes.

Ten animals of each species were exposed at each concentration level, and the LC_{50} with 95% confidence limits was calculated using the probit method of Finney (1971). The animals were observed frequently during the exposure and twice daily during the 14-day holding period, and visible signs of toxicity were recorded. All animals were weighed prior to exposure, and all survivors were also weighed on days 1, 2, 4, 7, and 14 postexposure.

A gross necropsy was performed on any animal that died as a result of exposure. The surviving animals were observed for 14 days, at which time they were sacrificed by halothane inhalation. Gross examination of lungs, liver, kidneys, heart, stomach, small and large intestine, spleen, and brain was performed on each animal. If signs of neurotoxicity (e.g., ataxia, paralysis) were observed, sections of spinal cord and sciatic nerve were also sampled for histological examination.

RESULTS

Oral Toxicity

Oral doses of TEP produced rapid breathing and tremors in the animals prior to death. Most deaths occurred within 24 h of dosing. Acute oral exposure of TEP resulted in LD_{50} values of 3.7 and 3.8 g/kg for male and female mice, respectively. TEP was slightly more toxic to rats with LD_{50} values of 2.5 and 1.8 g/kg for male and females, respectively. It was not necessary to orally dose male mice at 4.5 and 3.5 g/kg because sufficient dose levels and mortality ratios were available for the calculation of an LD_{50}. This situation also occurred with male and female rats. Surviving mice and rats demonstrated weight gains during the subsequent 14-day observation period.

Skin Irritation

A summary of the skin irritation effects of TEP is provided in Table 3.5.2-2. Using the interpretation of the NIOSH skin test ratings, a primary index score of 1.7 indicates that TEP is a mild irritant. The irritation effect of TEP was persistent; erythema was evident at the contact sites for 4 weeks posttreatment. However, the skin returned to normal appearance by 8 weeks posttreatment.

TABLE 3.5.2-2 SUMMARY OF RABBIT SKIN IRRITATION EFFECTS AFTER 4-H CONTACT WITH TRIETHYLPHOSPHITE

4 Hours	Symptoms 24 Hours	72 Hours
Very slight erythema (4). Very slight edema (3).	Well-defined erythema (6).	Well-defined erythema (6).

Primary irritation index score = 1.7
Classification: mild irritant
( ) Number of rabbits showing symptoms

Eye Irritation

Slightly constricted pupils were observed in all treated eyes of the nine rabbits following treatment with TEP. By 24 h, the pupils of the treated eyes were still slightly constricted and showed sluggish reaction to light. Two of the treated eyes showed a diffuse corneal opacity when stained with fluorescein. One of these had been washed following treatment while the other was unwashed. Irritation was not observed in the iris or conjunctivae. By 48 h, all pupils appeared normal and responded to light. The rabbit with the unwashed eye showed a diffuse corneal opacity at 48 h, but this effect disappeared by 72 h posttreatment.

Dermal Toxicity

Mortality occurred in both sexes of rabbits, of a ratio of 1/5 male and 2/5 females. Consequently, higher doses were administered in an attempt to achieve an LD_{50} value. Administration of 2.5 ml/kg body weight did not result in mortality of either sex. Eighty percent of the male rabbits died following a dermal dose of 3.0 ml/kg, while a similar dose resulted in only 10% mortality in the female rabbits. Higher doses of TEP were not administered to female rabbits because the 3.0 ml/kg dose level had already exceeded the normal upper-limit dose by 50%. Therefore, an LD_{50} value of 2.8 g/kg was determined for male rabbits only, the most susceptible animal.

Inhalation Toxicity

Chromatographs of the chamber atmosphere showed that approximately 10 to 30% of the aerosolized TEP decomposed during the exposures. A summary of the chamber GC data taken during the exposures is presented in Table 3.5.2-3. Ethanol (ETOH), diethylphosphite (DEP), and triethylphosphate (TEPA) were the main impurities seen in the exposure chamber samples. Several experiments were conducted to determine the accuracy of the chamber GC analysis. When water was added to TEP, the peaks with the retention times of ETOH and DEP increased. Also, when different quantities of water were added to TEP, the ETOH and DEP peaks changed in proportion to the amount of water added, while the ratio of the ETOH to DEP peak areas remained constant. Because the ETOH peak was not resolved from the diethyl ether solvent peak in the chamber samples, this provided a method to assess ethanol content of the chamber atmospheres.

TABLE 3.5.2-3. GC RESULTS OF CHAMBER ATMOSPHERES

Chamber Conc. (mg/m^3)	Average % Peak Areas for Each Exposure (min)			
	ETOH[a]	TEP	DEP[a]	TEPA[a]
2,135	2.00	88.8	5.55	0.00
4,121	2.30	90.5	6.40	0.90
8,837	3.99	83.5	11.05	2.05
12,980	3.66	84.8	10.15	1.35
16,397	4.30	82.2	11.90	1.85
14,077	3.92	83.4	10.85	2.18
13,039	4.48	74.35	12.40	10.9
11,414	5.27	74.28	14.60	11.66
9,882	4.60	65.44	12.74	17.36
8,418	4.87	63.48	13.48	18.20
9,149	4.52	65.95	12.52	21.25
8,336	5.23	75.1	14.75	1.97
6,861	6.09	75.1	16.88	2.12
5,975	6.97	71.8	19.30	1.91
3,350	7.16	72.2	19.86	1.83

[a] Peak identification by comparison of retention times with known standards.

A fivefold increase in the amount of TEPA occurred in the chamber GC samples from several consecutive exposures compared to the amount seen in the initial exposures (Table 3.5.2-3.). Using distilled diethyl ether as the impinger solvent to collect chamber samples thereafter indicated that the large increase in TEPA was due to an impurity in the ether (possibly peroxides). Therefore, it was concluded that no significant oxidation of the TEP was occurring in the chamber. This result was reproduced experimentally by using distilled ether.

Aerosol analysis was initially attempted using a Royco 227 particle counter; however, the amount of aerosol present exceeded the limits of this instrument. Therefore, subsequent analyses were conducted using an Andersen 8-stage cascade impactor. Aerosol analysis data are shown in Table 3.5.2-4.

A summary of the 6-h inhalation effects of TEP on male and female mice and rats is provided in Tables 3.5.2-5 and 3.5.2-6, respectively. TEP appeared to be slightly more toxic to male mice than female mice when the relative LC_{50} values were compared. The apparent increased toxicity was caused by a reversal in the mortality rate in a group of male mice exposed to 8336 mg/m^3. This reversal also precluded the calculation of confidence limits for the male mouse LC_{50}.

A similar reversal in mortality rate occurred in the female rats exposed to 12,980 mg/m^3 TEP. This reversal also resulted in the inability to calculate confidence limits.

TABLE 3.5.2-4. SUMMARY OF CHAMBER ANALYTICAL DATA

Target Conc.[a]	Aanlyzed Conc.[a]	Conc. SD (N = 12)	Aerosol Conc.[a]	Aerosol MMAD[c]	Aerosol Sigma G[d]
2,000	2,135	128	[b]	>5	[e]
4,000	4,121	363	3	3.5	[e]
8,000	8,837	528	161	1.6	2.2
12,000	12,980	851	429	1.6	2.0
15,000	16,397	1,143	567	1.6	2.0
12,500	14,077	1,485	348	1.6	2.0
11,500	13,039	1,442	395	1.7	2.0
13,500	11,414	633	373	1.8	2.0
10,000	9,892	721	288	1.9	1.9
8,000	8,418	452	160	1.8	2.0
9,000	9,149	709	258	2.0	2.0
8,800	8,836	813	292	2.1	2.0
7,500	6,861	725	181	1.7	1.9
6,000	5,975	499	230	1.9	2.0
3,500	3,350	229	51	1.8	2.0

[a] mg/m^3
[b] Aerosol concentration could not be calculated for this sample because of the large particle size.
[c] Mass median aerosol diameter.
[d] Geometric standard deviation of MMAD.
[e] These values were not determined because the Royco analyzer was used and it is not possible to calculate this value accurately.

TABLE 3.5.2-5. SUMMARY OF 6-HOUR INHALATION TOXICITY RESULTS FROM CD-1 MICE EXPOSED TO TEP

	Mortality Ratio	
Concentration, mg/m^3	Males	Females
9,892	9/10	9/10
9,149	10/10	6/10
8,418	2/10	0/10
8,336	10/10	----[a]
6,861	7/10	----[a]
5,975	4/10	----[a]
3,350	1/10	----[a]
LC_{50} (95% C.L.) mg/m^3 =	6203	9164
	(----)[b]	(8821-9469)

[a] Not done
[b] Cannot be calculated

TABLE 3.5.2-6. SUMMARY OF 6-HOUR INHALATION TOXICITY RESULTS FROM FISCHER-344 RATS EXPOSED TO TEP

Concentration, mg/m^3	Mortality Ratio	
	Males	Females
16,398	10/10	10/10
14,077	10/10	10/10
12,999	----[a]	10/10
12,980	5/10	1/10
11,444	----[a]	7/10
8,837	1/10	2/10
4,121	0/10	0/10
2,134	0/10	0/10
LC_{50} (95% C.L.) mg/m^3	11,620	11,063
	(10,083-12,761)	(----[b])

[a] Not done
[b] Cannot be calculated

DISCUSSION

TEP produced mild skin irritation (primary irritation index score of 1.7), which persisted for 4 weeks posttreatment. Instillation of TEP in the eyes of rabbits produced minimal effects that disappeared by 72 h. The dermal LD_{50} values for male and female rabbits of 2.8 and >3.0 g/kg, respectively, would classify this compound as relatively nonhazardous by the dermal route. These results indicate that TEP is less hazardous than QL, as QL proved to be corrosive by skin contact and the dermal LD_{50} values in male and female rabbits were less than 2.0 g/kg (Vernot *et al.*, 1984). TEP would be considered toxic through oral ingestion but its potency was not much different than that reported for QL.

The LC_{50} for inhalation of TEP (approximately 10,000 mg/m^3) indicated a lower order of toxicity than that reported for QL (male rats, male and female mice, 2520, 3502, and 2360 mg/m^3, respectively; Vernot *et al.*, 1985). Of the species tested, male mice appear to be the most susceptible to the inhalation effects of TEP.

REFERENCES

Campbell, K.I., E.L. George, L.L. Hall, and Stara, J.F. 1975. Dermal irritancy of metal compounds. *Arch. Environ. Health* 30:168-170.

Draize, J.H. 1959. Dermal toxicity. In: The Staff of the Division of Pharmacology of the Federal Food and Drug Administration, eds. *Appraisal of the Safety of Chemicals in Food, Drugs, and Cosmetics*, pp. 46-49. Austin, TX: The Editorial Committee of the Associates of Food and Drug Officials of the United States.

Finney, D.J. 1971. *Probit analysis.* 2nd Edition, p. 131. Cambridge, England: Cambridge University Press.

Vernot, E.H., J.D. MacEwen, E.R. Kinkead, J.R. Horton, J.D. Collins, W.J. Bashe, C.C. Haun, A.K. Roychowdhury, R.A. Salomon, and Olajos, E.J. 1984. Studies on the Acute Toxicity of O-ethyl-O'-(2-diisopropylaminoethyl)methylphosphonite (EDMP). CRDC-CR-84116. Aberdeen Proving Ground, MD: U.S. Army Armament, Munitions and Chemical Command.

3.5.3 Evaluation of the Inhalation Toxicity and the Irritation and Sensitization Potential of Chloropentafluorobenzene

E. R. Kinkead, W. J. Bashe, D. M. Brown, and S. S. Henry

INTRODUCTION

Chloropentafluorobenzene (CPFB) is a candidate material for use as a chemical warfare simulant for training purposes. Preliminary screening has indicated that CPFB provides good detectability for biological monitoring, desirable partitioning in biological tissues, acceptable physical properties, and relative biological inertness (Jepson *et al.*, 1985). For CPFB to be considered for use as a training simulant, however, it must be demonstrated as safe to humans.

This study was designed to evaluate the primary irritation hazard, sensitization potential, and acute inhalation toxicity of CPFB. Eye and skin irritation studies were conducted on albino rabbits, and sensitization tests were conducted on guinea pigs. The inhalation toxicity tests were performed with male rats because this species has been used in previous studies of this type.

In addition to the acute toxicity tests, metabolism of CPFB in rats will be measured after a single 4-h inhalation exposure. Blood and urine samples will be monitored for the concentration of CPFB and CPFB metabolites. The expired breath of rats receiving a single 1-h inhalation exposure to CPFB will also be analyzed to estimate the amount excreted by the respiratory system.

Suitable *in vitro* techniques are not available to replace animal testing of the type described by this protocol. The results obtained during these short-term exposure tests will be useful in establishing procedures and exposure concentrations for an eventual 90-day subchronic inhalation study.

MATERIALS AND METHODS

Test Agent

The CPFB used in this study was purchased from Aldrich Chemical Co., Milwaukee, WI. The physical properties of CPFB are shown in Table 3.5.3-1.

TABLE 3.5.3-1. PHYSICAL CHARACTERISTICS OF CHLOROPENTAFLUOROBENZENE

Chemical formula	C_6ClF_5
Molecular weight	202
Boiling point (°C)	117
Density (g/mL)	1.66
Vapor pressure (mmHg, 25°C)	14.1

Skin Irritation

The standard EPA patch-test method was utilized to determine the degree of primary skin irritation on the intact skin of six albino rabbits. The hair on the rabbits' backs and flanks was clipped as closely as possible 24 h prior to exposure to allow the skin to recover from any abrasion from the clipping. Undiluted test material (0.5 mL) was applied to the designated patch area, and covered by surgical gauze. The gauze was held in place with strips of surgical adhesive tape. The entire area was covered with polyethylene plastic wrap and secured with Elastoplast® tape. The patch remained in place on the rabbits for 4 h, after which the wrap and patch were carefully removed, and the test area evaluated for irritation using the Draize (1959) table as a reference standard. Readings were also made at 72 h after treatment. A primary index rating was calculated using the following formula:

$$\text{Primary Irritation Index} = \frac{\text{Total Reaction Score}}{(\text{No. of Rabbits})\,\text{Test Sites/Rabbit})\,(\text{Times Scored})}$$

The primary index score was interpreted using the NIOSH skin test ratings (Campbell *et al.*, 1975).

Eye Irritation

One-tenth of a milliliter of the test compound was applied to one eye of each of nine albino rabbits. The opposite eye was untreated and served as a control. The eyes were examined with fluorescein stain prior to use to ensure absence of lesions or injury. A topical anesthetic was instilled in the eyes of all rabbits, treated and control, approximately 2 min prior to application of the test substance. The treated eyes of six rabbits remained unwashed, while the treated eyes of the other three rabbits were flushed for 1 min with lukewarm water starting no sooner than 20-30 s after instillation of the test compound. Examinations for gross signs of eye irritation were made 1, 2, 3, 4, and 7 days following application. If an injury occurred, the animals were scored three times a week until the lesion subsided or was deemed irreversible. The irritative effects were scored according to the method of Draize (1959).

Skin Sensitization

CPFB was tested using a modification of the Maguire method (Maguire, 1973) as described by Horton *et al.* (1981). Ten male albino guinea pigs, Hartley strain, six to eight weeks old, were used in the sensitization test. An area on the back of each animal, directly above the forelegs, was clipped with electric clippers. The remaining fur was removed with a chemical depilatory on the morning of the first insult exposure. The test material (25% dilution in mineral oil) was applied to this area, and covered by gauze and dental dam. The gauze was secured with adhesive tape. The first insult patch was allowed to remain in place for two days. It was then removed, and a second similar application

was made. Two days later, this patch was removed, a total of 0.2 mL of Freund's adjuvant per animal was injected intradermally into two to three sites adjacent to the insult area, and a new test patch was applied. Three days later the final application was made; it remained for two days. A two-week induction period followed the removal of the final patch.

After the two-week induction period, the flanks of the animal were clipped and challenged with the test material on one side and the mineral oil vehicle on the other. The challenge applications were not occluded. The skin response at these sites was recorded at 24 and 48 h after application according to the evaluation method provided in Table 3.5.3-2. Any animal eliciting a score of 2 or more at the test solution challenge site at the 48-h scoring was rating a positive responder.

TABLE 3.5.3-2. GRADING OF SKIN REACTIONS IN THE MAGUIRE GUINEA PIG SENSITIZATION TEST

Erythema	Edema
0 – None	0 – None
1 – Very slight pink	1 – Very slight
2 – Slight pink	2 – Slight
3 – Moderate red	3 – Moderate
4 – Very red	4 – Marked

Acute Inhalation

Animals. Male and female Fischer-344 rats, female New Zealand White rabbits, and male albino guinea pigs, Hartley strain, were used in these studies. The animals were fed Purina Laboratory Chow and housed in an animal facility providing a 12-h light-dark cycle with controlled heat and humidity.

Generation and Analysis. CPFB vapor was generated by passing a known volume of air through an evaporator flask containing liquid CPFB. The resultant vapor was directed to the chamber input air, which was maintained at 12 air exchanges per hour. The chamber concentration of CPFB was continuously analyzed using a Miran 1A (Foxboro, CT) infrared analyzer equipped with a 12-cm gas cell.

Animal Exposure. Male and female Fischer-344 rats were used for the acute 4-h inhalation test. Testing was initiated by exposing five rats of each sex to an upper limit concentration of 5 mg/L. If no mortality occurred during the 14-day observation period, no further testing would be conducted.

The rats were observed frequently during the day of exposure and twice daily during the 14-day holding period. Body weights of all rats were obtained at the time of exposure and on days 1, 2, 4, 7, 10 and 14 following exposure.

RESULTS

Skin Irritation

A group of six female rabbits was used to measure the degree of primary dermal irritation of intact skin to CPFB. The compound did not produce primary skin irritation on four of the six rabbits. Mild erythema was observed on the skin of two rabbits at 24 h postexposure. One of these showed no signs of irritation at 48 h; however, the second rabbit demonstrated an intensified reaction at 48 and 72 h. Diffuse epidermal sloughing of the skin of this rabbit was noted at one week posttreatment.

Eye Irritation

During the examination of rabbit eyes 4 h following treatment with CPFB, mild conjunctival irritation was observed in two of the washed and two of the unwashed eyes. Complete recovery of irritative effects occurred by 24 h. Subsequent observations through one week posttreatment revealed no delayed effects.

Skin Sensitization

A group of 10 guinea pigs was tested for sensitization response to CPFB. The guinea pigs demonstrated no sensitization reaction to CPFB at either evaluation period.

Acute Inhalation

The mean concentration of CPFB vapor during the 4-h period was 4.84 mg/L. None of the exposed rats died during exposure or during the 14-day observation period. Mean body weights of the male rats, following an initial decrease after exposure, showed a gradual increase through the 14-day observation period. The female rats, however, did not regain their initial mean weight until 14 days postexposure.

DISCUSSION

Applications of undiluted CPFB to intact rabbit skin produced a diffuse epidermal sloughing one week postapplication. One rabbit showed mild, but reversible, irritation while the remaining four rabbits demonstrated no dermal irritation response to CPFB. These results would indicate that CPFB is a mild skin irritant. Rabbit skin is considered to be more sensitive than human skin (Nixon *et al.*, 1975), so it is unlikely that CPFB would produce a skin response in humans greater than that demonstrated in the rabbit.

Application of the undiluted compound into the rabbits' eyes produced mild conjunctival irritation that dissipated completely by 24 h. These results would indicate that CPFB is a mild eye irritant.

The skin sensitization test was designed to evaluate the potential of a material to act as an antigen. Application of small quantities of antigenic material over a period of time induces antibody production. The induction potential can then be evaluated by grading the response to a challenge administration of the material. The fact that none of the guinea pigs responded to the challenge application of CPFB indicates an absence of antibody production and therefore a lack of sensitization potential.

CPFB vapor poses no serious hazard by the inhalation route, as all rats survived a 4-h exposure of 4.84 mg/L, a concentration unlikely to be encountered in the field. CPFB vapor would be considered safe for use as a training simulant with a possibility of minor skin and eye irritation.

REFERENCES

Campbell, K.I., E.L. George, L.L. Hale, and J.F. Stara. 1975. Dermal irritancy of metal compounds. *Arch. Environ. Health* 30:168-170.

Draize, J.H. 1959. Dermal toxicity. In: The Staff of the Division of Pharmacology of the Federal Food and Drug Administration, eds. *Appraisal of the Safety of Chemicals in Food, Drugs, and Cosmetics*, pp. 46-49. Austin, TX: The Editorial Committee of the Associates of Food and Drug Officials of the United States.

Horton, J.R., J.D. MacEwen, E.H. Vernot, and A. Hall. 1981. *Comparisons of Skin Sensitization Methods: Landsteiner, Maguire and Guinea Pig Maximization.* AFAMRL-TR-81-131 (ADA-110633). Wright-Patterson Air Force Base, OH: Air Force Aerospace Medical Research Laboratory.

Jepson, G.W., H.J. Clewell, and M.E. Andersen. 1985. A rapid, physiologically based method for evaluating candidate chemical warfare agent uptake simulants, Air Force Technical Report AAMRL-TR-85-045 (ADA-159152). Wright-Patterson Air Force Base, OH: Harry G. Armstrong Aerospace Medical Research Laboratory.

Maguire, H.C. 1973. The bioassay of contact allergens in the guinea pig. *J. Soc. Cosmet. Chem.* 24:151-162.

Nixon, G.A., C.A. Tyson, and W.C. Wertz. 1975. Interspecies comparison of skin irritancy. *Toxicol. Appl. Pharmacol.* 31:481-490.

3.5.4 *In Vitro* Biological Activity of Chloropentafluorobenzene

R. S. Kutzman

INTRODUCTION

The Air Force is investigating chloropentafluorobenzene (CPFB) as a potential compound for use as an uptake simulant of chemical warfare agents with relatively high vapor pressures. This simulant could be used in the field to determine whether protective measures taken by troops exposed to chemical warfare agents are adequate. CPFB has chemical and physical properties consistent with the requirements for such a simulant. For CPFB to be considered for use as a training simulant, however, it must be demonstrated as safe for humans.

Studies have been undertaken by the *In Vitro* Toxicology Section of NSI-ES (Research Triangle Park, NC) to investigate the genetic toxicity potential of CPFB. These studies were developed in two phases. The first was designed to determine the solubility of CPFB in the media necessary to conduct the cell culture studies. Once suitable solubilization procedures are developed, protocols will be written for the *in vitro* genotoxicity studies.

METHODS

Solubility Studies

Commercial CPFB, 99% pure, was obtained from Aldrich Chemical Company. All dilutions were made in glass or plastic dishes using glass pipets. Glass dishes were placed into a 37°C vented incubator with a water-saturated atmosphere of 5% CO_2 in air. The dishes were held for 10 days before discarding. All experiments were visually examined every working day, and observations were made and recorded. In experiments with plastic dishes, blistering was observed immediately after treatment with CPFB; these samples could not be maintained within the incubator.

Experimental Design

To determine the potential for CPFB to induce genetic damage, a protocol was developed that utilized a variety of *in vitro* genetic and cytogenetic assays. CPFB will be tested for mutagenic potential by employing auxotrophic strains of Salmonella developed as a screen for potential carcinogens. However, bacterial cells do not contain the complement of metabolic enzymes required to activate procarcinogens/mutagens. Therefore, exogenous metabolic enzymes obtained from rat livers from animals pretreated with Aroclor 1254 will be added to the culture media for the testing of CPFB.

A Chinese hamster ovary cell assay will be utilized to detect phenotypic changes that result from small changes in nucleotide sequence or other genetic alterations that result in the loss of

enzyme activity at the hypoxanthine-guanine phosphoribosyl transferase locus. Metabolic activation using exogenous rat liver enzymes will be employed in this assay because some chemicals are not active until metabolized.

Chinese hamster ovary cells will also be used for the sister chromatid exchange assay. This assay depends upon the detection of the exchange of DNA from sister chromatids within the chromosome. Such exchanges apparently require the breakage and rejoining of DNA; these changes are visualized by the differential incorporation of fluorescent nucleotides into the newly synthesized DNA strand during replication. Cytogenetic analysis of chemically treated cells will permit the determination of the clastogenic potential of the chemical with which the cells were treated. As in the other *in vitro* assays already described, a metabolizing system will be provided by conducting the test both with and without the addition of exogenous liver homogenate from animals pretreated with Aroclor 1254.

These studies will include another measure of DNA damage in mammalian cells by indirectly measuring the incorporation of radioactive DNA precursors into repair sites on the DNA molecule. In this assay, freshly isolated rat hepatocytes will be used as target cells because of their xenobiotic metabolizing capabilities.

Finally, a cell transformation assay that permits the *in vitro* evaluation of the carcinogenic potential of CPFB (BALB/c-3T3 transformation assay) will be conducted. In this assay, the phenotypic appearance of morphologically altered cells has been correlated with the potential of these cells in subsequent passages to express phenotypes that grow in soft agar or demonstrate tumor formation in syngenic hosts.

RESULTS

Solubility Studies

Table 3.5.4-1 shows the results when Eagle's minimum essential medium (EMEM) was used as a solvent for CPFB. When CPFB was diluted by EMEM containing 10% fetal bovine serum in 60-mm glass tissue culture dishes, a precipitate was observed at concentrations of 400 mg/mL or greater. No precipitate was observed at any of the concentrations tested when CPFB was diluted in glass dishes with EMEM not containing serum. A precipitate was noted at concentrations as low as 1 mg/mL CPFB when plastic tissue culture dishes were used. The data indicate that CPFB is soluble in EMEM + 10% FBS at concentrations up to 200 mg/mL when glass dishes are used. The identity of the white amorphous precipitate is unknown. Experiment III represents a typical experimental dose range of CPFB in media using cytotoxicity data from the previous studies. When glass was used, no precipitate was seen.

TABLE 3.5.4-1. SOLUBILITY DATA ON CHLOROPENTAFLUOROBENZENE IN GROWTH MEDIUM

Solvent	Experiment	CPFC[a] (mg/mL)	FSB[b]	G/P[c]	Observation
None	I	600	+	G	Moderate amount of precipitate
		600	–	G	No precipitate
		400	+	G	Small amount of precipitate
		400	–	G	No precipitate
		200	+	G	No precipitate
		200	–	G	No precipitate
	II[d]	3	+	P	Large amount of precipitate
		3	–	P	Large amount of precipitate
		2	+	P	Moderate amount of percipitate
		2	–	P	Moderate amount of percipitate
		1	+	P	Very small amount of precipitate
		1	–	P	Very small amount of precipitate
	III[e]	0.784	+	G	No precipitate
		0.0784	+	G	No precipitate
		0.00784	+	G	No precipitate
		0.000784	+	G	No precipitate
		0.0000784	+	G	No precipitate

[a] CPFB was diluted into Eagle's minimum essential medium (EMEM). Serial dilutions were used to yield the specified concentrations of CPFB.
[b] Fetal bovine serum (FBS), when added, was at a final concentration of 10% in EMEM.
[c] Glass (G) or plastic (P) 60-mm tissue culture dishes.
[d] Aliquots of the above samples (Experiment I) were diluted further to yield these CPFB concentrations.
[e] An aliquot of the 3-mg/mL CPFB sample (+ FBS) from Experiment II was diluted further to attain these concentrations.

Table 3.5.4-2 shows the results of experiments in which dimethyl sulfoxide (DMSO) was used as a solvent for CPFB. At concentrations greater than 200 mg/mL, CPFB caused either blistering of plastic dishes or formation of a precipitate in glass dishes when serum was present in the medium; no blistering or precipitate was observed in plastic or glass dishes at approximately 200 mg/mL CPFB. Similar results were observed when acetone was used as the solvent (Table 3.5.4-3).

TABLE 3.5.4-2. SOLUBILITY DATA ON CHLOROPENTAFLUOROBENZENE IN DMSO

Solvent	Experiment	CPFB[a] (mg/mL)	EMEM + 10% FBS	G/P[b]	Observation
DMSO	I	600	--	P	Blistering of dishes
		400	--	P	Blistering of dishes
		200	--	P	No blistering; no precipitate
	II	600	--	G	Very thick layer of precipitate
		400	--	G	Thick layer of precipitate
		200	--	G	Very small amount of precipitate

(continued)

TABLE 3.5.4-2. (Continued)

Solvent	Experiment	CPFB[a] (mg/mL)	EMEM + 10% FBS	G/P[b]	Observation
	III	156.8	--	G	No precipitate
		15.68	--	G	No precipitate
		1.568	--	G	No precipitate
		0.1568	--	G	No precipitate
		0.01568	--	G	No precipitate
	IV[c]	0.784	+	G	No precipitate
		0.0784	+	G	No precipitate
		0.00784	+	G	No precipitate
		0.000784	+	G	No precipitate
		0.0000784	+	G	No precipitate

[a] CPFB was diluted into dimethyl sulfoxide (DMSO, 99.9% pure) to yield the described concentrations.
[b] Glass (G) or plastic (P) 60-mm tissue culture dishes.
[c] 1:200 dilutions were made from corresponding stocks of CPFB in DMSO (Experiment III) into EMEM + 10% FBS. For example, the 156.8 mg/mL DMSO stock was diluted 1:200 using EMEM + 10% FBS to yield 0.784 mg/mL, and the 15.68 mg/mL stock was diluted to give 0.0784 mg/mL in medium, etc.

TABLE 3.5.4-3. SOLUBILITY DATA ON CHLOROPENTAFLUOROBENZENE IN ACETONE

Solvent	Experiment	CPFB[a] (mg/mL)	EMEM + 10% FBS	G/P[b]	Observation
Acetone	I	600	--	P	Blistering of dishes
		400	--	P	Blistering of dishes
		200	--	P	Blistering of dishes
	II	600	--	G	Large amount of precipitate
		400	--	G	Small amount of precipitate
		200	--	G	No precipitate
	III	156.8	--	G	No precipitate
		15.68	--	G	No precipitate
		1.568	--	G	No precipitate
		0.1568	--	G	No precipitate
	IV[c]	0.784	+	G	No precipitate
		0.0784	+	G	No precipitate
		0.00784	+	G	No precipitate
		0.000784	+	G	No precipitate
		0.0000784	+	G	No precipitate

[a] CPFB was diluted serially into acetone (99.5% pure) to yield the described concentrations.
[b] Glass (G) or plastic (P) 60-mm tissue culture dishes.
[c] 1:200 dilutions were made from corresponding stocks of CPFB in acetone (Experiment III) into EMEM + 10% FBS. For example, the 156.8 mg/mL acetone stock was diluted 1:200 using EMEM + 10% FBS to yield 0.784 mg/mL, and the 15.68 mg/mL stock was diluted to give 0.0784 mg/mL in medium, etc.

A number of other organic solvents were also used to investigate the solubility of CPFB (Tables 3.5.4-4 and 3.5.4-5). All concentrations of CPFB tested in these studies caused plastic dishes to blister. When tested in glass dishes, precipitates were formed at higher CPFB concentrations, and globules formed when serum was present. The data indicate that higher concentrations of soluble CPFB could not be achieved with these organic solvents than was possible with EMEM, DMSO, or acetone.

TABLE 3.5.4-4. SOLUBILITY DATA ON CHLOROPENTAFLUOROBENZENE IN EGDME AND EGDEE

Solvent	Experiment	CPFB[a] (mg/mL)	EMEM + 10% FBS	G/P[b]	Observation
EGDME	I	156.8	--	P	Blistering of dishes
		15.68	--	P	Blistering of dishes
		1.568	--	P	Blistering of dishes
		0.1568	--	P	Blistering of dishes
	II[c]	0.784	+	G	Large amount of glubules
		0.0784	+	G	Moderate amount of globules
		0.00784	+	G	Small amount of globules
		0.000784	+	G	No globules
		0.0000784	+	G	No globules
EGDEE	I	156.8	--	P	Blistering of dishes
		15.68	--	P	Blistering of dishes
		1.568	--	P	Blistering of dishes
		0.1568	--	P	Blistering of dishes
		0.01568	--	P	Blistering of dishes
	II[c]	0.784	+	G	Small amount of precipitate
		0.0784	+	G	Very small amount of precipitate
		0.00784	+	G	No precipitate
		0.000784	+	G	No precipitate
		0.0000784		G	No precipitate

[a] CPFB was diluted into EGDME (ethylene glycol dimethyl ether, 99 + % pure) or EGDEE (ethylene glycol diethyl ether, 95% pure). Serial dilutions were completed to yield the specified concentrations.

[b] Glass (G) or plastic (P) 60-mm tissue culture dishes.

[c] 1:200 dilutions were made from corresponding stocks of CPFB in EGDME or EGDEE (Experiment I) into EMEM + 10% FBS For example, the 156.8 mg/mL EGDME stock was diluted 1:200 using EMEM + 10% FBS to yield 0.784 mg/mL, and the 15.68 mg/mL stock was diluted to give 0.0784 mg/mL in medium, etc.

TABLE 3.5.4-5. SOLUBILITY DATA ON CHLOROPENTAFLUOROBENZENE IN OTHER ORGANIC SOLVENTS

Solvent	Experiment	CPFB[a] (mg/mL)	EMEM + 10% FBS	G/P[a]	Observation
Hexane[b]	I	156.8	--	G	No precipitate
		15.68	--	G	No precipitate
		1.568	--	G	No precipitate
		0.1568	--	G	No precipitate
		0.01568	--	G	No precipitate
	II	0.784	+	G	No precipitate
		0.0784	+	G	No precipitate
		0.00784	+	G	No precipitate
		0.000784	+	G	No precipitate
		0.0000784	+	G	No precipitate
Pluronic Polyol F-127[c]	I	156.8	--	G	No precipitate
		15.68	--	G	No precipitate
		1.568	--	G	No precipitate
		0.1568	--	G	No precipitate
		0.01568	--	G	No precipitate
Pluronic Polyol F-68[d]	II	0.784	+	G	No precipitate
		0.0784	+	G	No precipitate
		0.00784	+	G	No precipitate
		0.000784	+	G	No precipitate
		0.0000784	+	G	No precipitate
THF[e]	I	156.8	--	G	No precipitate
		15.68	--	G	No precipitate
		1.568	--	G	No precipitate
		0.1568	--	G	No precipitate
		0.01568	--	G	No precipitate
THF[f]	II	0.784	+	G	Small amount precipitate
		0.0784	+	G	No precipitate
		0.00784	+	G	No precipitate
		0.000784	+	G	No precipitate
		0.0000784	+	G	No precipitate

[a] Glass (G) or plastic (P) 60-mm tissue culture dishes.

[b] CPFB was diluted into hexane (99 9 + % pure). Serial dilutions were completed to yield the specified concentrations.

[c] Pluronic polyol F-127 was dissolved 25%/75% (W/W) in ethanol; CPFB was diluted into F-127 to yield the specified concentrations.

[d] Pluronic polyol F-68 was dissolved in sterile distilled water to yield a final concentration of 5%. CPFB was then added and serial dilutions completed to give the described concentrations.

[e] CPFB was added to THF (tetrahydrofuran, 99.9% pure). Serial dilutions were done to yield the specified concentrations

[f] 1:200 dilutions were made from corresponding stocks of CPFB in each solvent into EMEM + 10% FBS. For example, the 156.8 mg/mL DMSO stock was diluted 1:200 using EMEM + 10% FBS to yield 0.784 mg/mL, and the 15.68 mg/mL stock was diluted to give 0.0784 mg/mL in medium, etc.

The white precipitate, which appeared as a white powder on the bottom of the glass dishes, remained even after rapid vortexing. The blistering observed in plastic dishes resulted in the immediate formation of a cloudy, white layer. The globules observed when CPFB was diluted into the organic solvent ethylene glycol dimethyl ether appeared as round beads, resembling oil droplets, throughout the dish.

Additional potential solvents were investigated with the following results. CPFB was soluble in hexane at all concentrations tested (up to 156.8 mg/mL). No precipitate formed in dilutions (1:200) of these stocks in EMEM + 10% FBS. Two pluronic polyols, F-127 and F-68, were tested as possible solvents. Only F-68 caused precipitate formation at the highest concentration tested. However, when F-68 was diluted with media, no precipitates formed. Tetrahydrofuran (THF) was also tested as a solvent for CPFB. CPFB was soluble in THF at all concentrations tested up to 156.8 mg/mL. Dilutions (1:200) into media produced a small amount of precipitate only at the highest concentration tested.

DISCUSSION

Because CPFB damaged the plastic dishes they should not be used in this type of testing. When samples were placed in glass dishes, the CPFB did not precipitate unless serum was present in EMEM, and this only occurred at higher concentrations. At lower concentrations of CPFB, no precipitate formed. These results indicate that CPFB can be tested at up to 200 mg/mL using glass dishes for *in vitro* genotoxicity assays. This is an unrealistic concentration; therefore, the highest concentrations used for genotoxicity testing will be governed by findings on cytotoxicity assays using the cells to be employed in the individual tests.

3.5.5 Protocol Development For Evaluation of Long-Term, Low-Level CW Agent Exposures

R. S. Kutzman

INTRODUCTION

The paucity of credible data on the effects of extended exposure to sublethal concentrations of chemical warfare (CW) agents has affected equipment specifications, threat assessment, treatment protocols, and operational procedures implemented by the DOD. For these reasons, low-level exposure criteria are of concern. In an effort to identify areas central to understanding physiological responses to long-term, low-level effects of CW agents, several data gaps were identified. Some examples were long-term, low-level effects of CW agents on animal models; effects of repeated exposures; methods for extrapolating animal models to humans; methods for quantitation of absorbed agent dose; and combined effects of different CW agents.

In order to provide some of the needed data, protocols were required for experiments that would establish miotic standards for sarin and soman. In addition to the need for establishing miotic standards, protocols were also needed to govern studies to establish inhalation standards for soman. These studies were to include daily blood cholinesterase determinations and blood chemistries as well as an extensive complement of postmortem or recovery data.

METHODS

The development of these protocols was subcontracted to Battelle Columbus Laboratories, where the staff has a history of work with CW agents for the DOD. The development of these protocols was divided into two phases. In the first phase, Battelle was to provide outline protocols. These would be reviewed by Air Force and NSI-ES personnel. After clarification of any points in the outline protocols and determination that the final protocol should be adequate for conduct of the studies, the second phase would be to develop detailed protocols.

Four draft protocols were received for review: two separate versions of a protocol to establish miotic standards for sarin and soman; and two versions of a protocol to establish inhalation standards, one utilizing dogs and the other utilizing nonhuman primates as the test species. The draft protocols were reviewed by the Air Force and NSI-ES, and the drafts of the final protocols have been distributed to the Air Force for review.

Experimental Design

The protocols, designed to aid in the establishment of miotic standards for sarin and soman, utilize whole-body exposure of young adult cynomolgus monkeys. Animals will be exposed to

concentration x time products ranging from 0 (controls) to 20 mg/min/m^3, with exposure concentrations as high as 2000 µg/m^3 and exposure durations ranging from 2 to 1200 min per day for 5 days. The miotic assessments require examination by a qualified veterinarian with documented training in ophthalmoscopy. Measurements will be made of the diameter of each animal's pupil before the first exposure to the test agent and again as soon as possible (within 30 min) after the end of each daily exposure, 8 h after the start of the first exposure, and every 8 h thereafter for five days. For animals monitored for recovery from exposure, additional evaluations will be conducted at 24, 72, and 168 h following the last exposure and, if necessary, at weekly intervals until all miotic symptoms disappear. At the same time that these assessments are made, a general examination of the eye will be performed to assess whether any pathology has been caused by the continuous or repeated low-level exposures. These examinations will specifically include the conjunctivae, cornea, iris, lens, and anterior fundus.

Repeated whole-body inhalation exposures will be used to establish inhalation standards for soman. These studies will utilize young adult beagle dogs and young adult cynomolgus monkeys. These test species were selected because of the extensive data base available on the species for the inhalation effects of soman. The objective of the study is to determine the toxicological effects of repeated inhalation exposure to soman as a function of total exposure dose, dose rate, and daily exposure duration to provide data for extrapolation to man. The animals will be whole-body exposed to soman over a range of concentrations and daily exposure times for 28 days. Groups of dogs will be sacrificed on days 0, 7, and 28 following exposure. In-life toxicological evaluation will include daily clinical observations and pre- and postexposure analysis of blood esterase activity. Hematology and clinical chemistry data will be collected prior to treatment, at the final exposure, and on the day of sacrifice. In addition, operant behavioral evaluations will be performed before the study, immediately following each daily exposure, and again three times during the recovery period. Physiologic measurements of cardiopulmonary function will be conducted before the start of each exposure (baseline), after the exposure period has concluded, and prior to terminal sacrifice of the animals after the 28-day recovery period. Postmortem evaluations will include organ weights and esterase activities from selected tissues, as well as microscopic examination of scheduled tissues.

3.5.6 Pharmacockinetic and Pharmacodynamic Validation of the Organophosphate Model

J. L. Cramer and R. B. Conolly

INTRODUCTION

A kinetic model for *in vivo* esterase inhibition by organophosphates is being developed by the Air Force for use in risk assessment and management of organophosphate (OP) exposures in civilian and military circumstances. This model can simulate the time course of inhibition of OP-susceptible esterases such as acetylcholinesterase and carboxylesterase.

The goal of this work is to collect pharmacokinetic and pharmacodynamic data on diisopropylfluorophosphate (DFP). Rats will be exposed to DFP by inhalation, by gavage, and dermally. These data will be used for validation of the OP model. OPs are important economic poisons (pesticides, herbicides) and CW agents. The model would be valuable for OP risk assessment and for the study of OP toxicities. Another important aspect of the OP model is its qualitative difference from the physiologically based pharmacokinetic models. It is expected that this model will reach segments of the scientific community that have not yet learned about biologically based mathematical models as research and regulatory tools.

MATERIALS AND METHODS

DFP

Experimental Approach. The time course of DFP in rat blood will be measured after intravenous (IV) dosing and inhalation exposure. This information will be used to determine appropriate DFP levels for time-course studies of enzymatic activity in blood and selected tissues.

AChE, BChE, and CE activity in blood, brain, liver, kidney, and lung will be measured at five IV dose levels and five inhalation exposure concentrations. For each exposure condition, six time intervals between 0 and 30 min will be used. It will be possible to remove either the blood and brain or the liver, kidney, and lungs from any one animal; therefore, two rats will be required to collect a complete sample at a given time point. Up to six replications of this experiment may be necessary depending on data variability. Tissues will be excised and dropped into liquid nitrogen to stop metabolic activity.

AChE activitiy in blood, brain, liver, kidney, lung, and PNS will be determined following inhalation exposure and IV dosing. Using this method the substrate, acetylthiocholine, is hydrolyzed by AChE to form thiocholine, which in turn reacts with dithionitrobenzoic acid to form the yellow-colored dithionitrobenzoic acid anion. The rate of formation of this yellow-colored anion, which has a maximum absorbance at 412 nm, and the subsequent change in absorbance, are directly proportional to the rate of AChE hydrolysis. Because the rate of hydrolysis is related to the amount

of enzyme available, it is possible to determine the amount of enzyme activity in that tissue by measuring the rates of hydrolysis (velocities) of the substrate upon addition of a small amount of tissue homogenate. By comparing the enzyme activities after a known duration of exposure to the time of dose, it is possible to obtain a time-course profile of enzymatic depression.

Biomolecular rate constants for enzyme inhibition will be determined as follows. Assays for AChE, BChE, and CE activity will be run on homogenates of blood, liver, kidney, lung, and PNS at three concentrations of DFP. Enzymatic activity will be assayed at 6 time intervals between 0 and 0.5 min. Up to six replications of each measuremnt will be made.

Other aspects of the experimental approach are under development.

When studying highly toxic materials such as DFP, it is essential that both the exposure apparatus (e.g., inhalation chamber, cage for dermal exposures) and the analytical apparatus (gas chromatograph) are contained within the same well-ventilated areas. Consequently, any leaks of toxicant from the experimental apparatus or during the process of transferring a sample from the exposure apparatus to the analytical instrument can be contained.

RESULTS/DISCUSSION

Most of the esterase assay development work needed for this project has been completed. The experimental phase of the project will start once the exposure facility described above is functional.

REFERENCES

Chemnitius, J.M., K.H. Haselmeyer, and R. Zech. 1982. Identification of isoenzymes in cholinesterase preparations using kinetic data of organophosphate inhibition. ***Analytical Biochem.*** 125:442-451.

Ellman, G.L., K.D. Courtney, V. Andres, Jr. and R.M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. ***Biochem. Pharmacol.*** 7:88-95.

Jepson, G.W. 1986. A kinetic model for acetylcholinesterase inhibition by diisopropylfluorophosphate in crude rat brain homogenate. Thesis. Wright State University, Dayton, OH.

Kato, T. and K. Shimotohno. 1984. Estimation of kinetic parameters for substrate and inhibitor in a reaction with an enzyme sample containing different types of Inhibitor. *Biochemica et Biophysica Acta.* 801:157-162.

Smith, S.J. 1983. Detection methods for highly toxic organophosphonates. *Talania.* 30:725-739.

SECTION 4

EXPOSURE AND FACILITIES ENGINEERING

4.1 FACILITIES ENGINEERING

D.W. Brade

During this first year of NSI-ES's operation of the THRU, a major effort of facilities engineering has been the development of facility modifications to permit the expansion of the THRU scientific program. Early activities involved inspection and evaluation of the equipment. Inspection of the dormant equipment led to the discovery of frozen bearings, leaky plugs, malfunctioning valves, and inadequate blower capacity for scrubbers. This equipment has been repaired, and/or rebuilt; where possible, it has been upgraded to prevent problems in future maintenance.

Initial operations of the Thomas Domes indicated the need for modifications in three areas. The spring-loaded mechanism, by which the access hatch covers were opened and closed, frequently malfunctioned, allowing the heavy covers to fall open and pose a risk of personal injury. The system was redesigned using a compressed air cylinder and shock absorbers to correct this condition. Now after releasing the safety bolts, the technician can, from a safe distance, push a button to cause the cover to open or close slowly.

The primary air supply for the domes was found to be inoperable. A group of air-handling specialists made a thorough inspection, and corrective action was recommended. Worn-out controls should be replaced and other controls adjusted. Airflow baffling in the system was also adjusted. While assisting in analyzing the mechanical and electrical problems, the maintenance staff became more familiar with this equipment.

The third major problem was with the waste discharge control valves, which were corroded, difficult to operate, and leaking. When the Thomas Domes are operated below atmospheric pressure, these valves both aid in the disposal of accumulated waste and form a seal when closed. This seal prevents leaks when a vacuum is created in the domes. New corrosion-resistant valves made of stainless steel were installed. In addition, the vacuum pumps and motors for the domes were rebuilt.

Inspection of the in-house compressed air system revealed that the main compressor, a Sullair rotary screw, was old and had many worn parts, including the main components of the air modules. The compressor is being repaired. Because of its condition, however, the compressor will no longer

be used as the main air supply. A new unit has been installed and, after repairs are completed, the Sullair will function as a backup compressor. The existing backup compressors are no longer capable of supplying the volume of compressed air used in Building 79, and removal should be considered. A new compressed air filtration system has been purchased and will provide clean, breathable air to dome Rooms A and B and the new ambient lab, as well as the other laboratories in Building 79 requiring compressed air.

The general toxicology lab (ambient lab) is being remodeled under Air Force contract. NSI-ES's plans will augment existing equipment and provide a regulated laboratory environment suitable for conducting toxicology research. Ultimately, the laboratory will be equipped with two isolation units to be used for highly hazardous materials. The laboratory can be configured to contain 12 inhalation exposures, or fewer chambers and the desired mix of research stations. A stand-alone ventilation system will provide clean, filtered air for inhalation studies. All exhaust air will be decontaminated before release, and redundant equipment will automatically operate in the event of an equipment failure. Six exposure chambers of a new design have been ordered. A washing system for the exposure chambers is being designed to make the task of cleaning the chambers as easy and thorough as possible.

The prototype machine shop was modernized and expanded. The Bridgeport mill was upgraded by adding a digital control system to control the cuts and travel. The old drill press and metal lathe were replaced with newer models. The shop was expanded by adding a metal shear, a surface grinder, a bench grinder, a sharpener for drill bits, a surface plate for use in making accurate measurements, plus accessories for these machines. This modernization has enabled NSI-ES personnel to fabricate more complicated instruments than was previously possible.

The supply of tooling used by the maintenance personnel was evaluated and subsequently expanded. Hand tools were added, along with special tooling such as a sandblaster, pipe threader, and metal-cutting band saw to enhance the capabilities of the maintenance personnel. With this expanded capability NSI-ES maintenance personnel can perform all the mechanical repairs needed in the operation of the THRU.

To reduce the noise level in some of the mechanical rooms, the possibility of soundproofing the rooms is being investigated. This may improve the productivity of the personnel working in these surroundings.

4.2 TOXICANT DISTRIBUTION IN THE THOMAS DOMES

R. L. Carpenter, E. C. Kimmel, C. D. Flemming, and C. R. Doarn

INTRODUCTION

When conducting inhalation toxicology studies, it is desirable to expose all the animals in the study to the same concentration of test agent. If the concentration of the test agent and the physical properties are uniform in the exposure apparatus, then the variation in inhaled dose of the test agent depends only on the variation in respiratory minute volume of the individual animals. This should result in the dose being more uniformly deposited among the animals. Evidence that uniform exposure concentrations reduces variability in inhaled dose is given in a paper by Heminway *et al.* (1982); in this study the animals were carefully permuted through selected positions in an inhalation chamber during an exposure. The animals that were rotated through the chamber positions exhibited significantly lower variations in inhaled dose than the animals that remained stationary (Heminway *et al.*, 1983). A knowledge of chamber distribution is necessary for selecting a chamber sampling point that is representative of the average chamber concentration for the purpose of measuring exposure concentration during an inhalation exposure. For these reasons, modern inhalation toxicology practice includes characterizing the concentration of the test agent and other relevant properties of test agents prior to conducting inhalation exposures.

The Toxic Hazards Division of the AAMRL has eight large exposure chambers known as the Thomas Domes (Figure 4.2-1). The domes can be used to expose animals to potential toxicants under environmental conditions simulating those of high altitudes and space flight. To gain an understanding of the operation of the Thomas Domes, researchers conducted test agent distribution studies within these exposure systems. We have analyzed the time taken for test agent concentration to reach its equilibrium value when a test agent is introduced into the airflow through the Thomas Domes. Additionally, the time taken for the test article concentration to fall to zero upon shut down of the domes has been measured. The degree of mixing between the air in the dome and newly introduced air can be calculated from a knowledge of the rise and fall characteristics of the dome exhaust concentration (Heminway *et al.*, 1985; Cholette and Cloutier, 1959).

The spatial distribution of the test article introduced into the domes has been determined for gaseous test articles. The hydrocarbon analyzers available to us will obtain data at a much higher rate than normal aerosol concentration measurement equipment. This high rate of data acquisition has allowed us to measure the propane concentration at sets of 50 points in a grid of 250 points. By analyzing these data we can determine how many measurement points are needed to characterize

the distribution of test articles within the domes. Finally, leak rate measurement methodology has been applied to operation of the domes to determine how much air from the room leaks into the domes during operation.

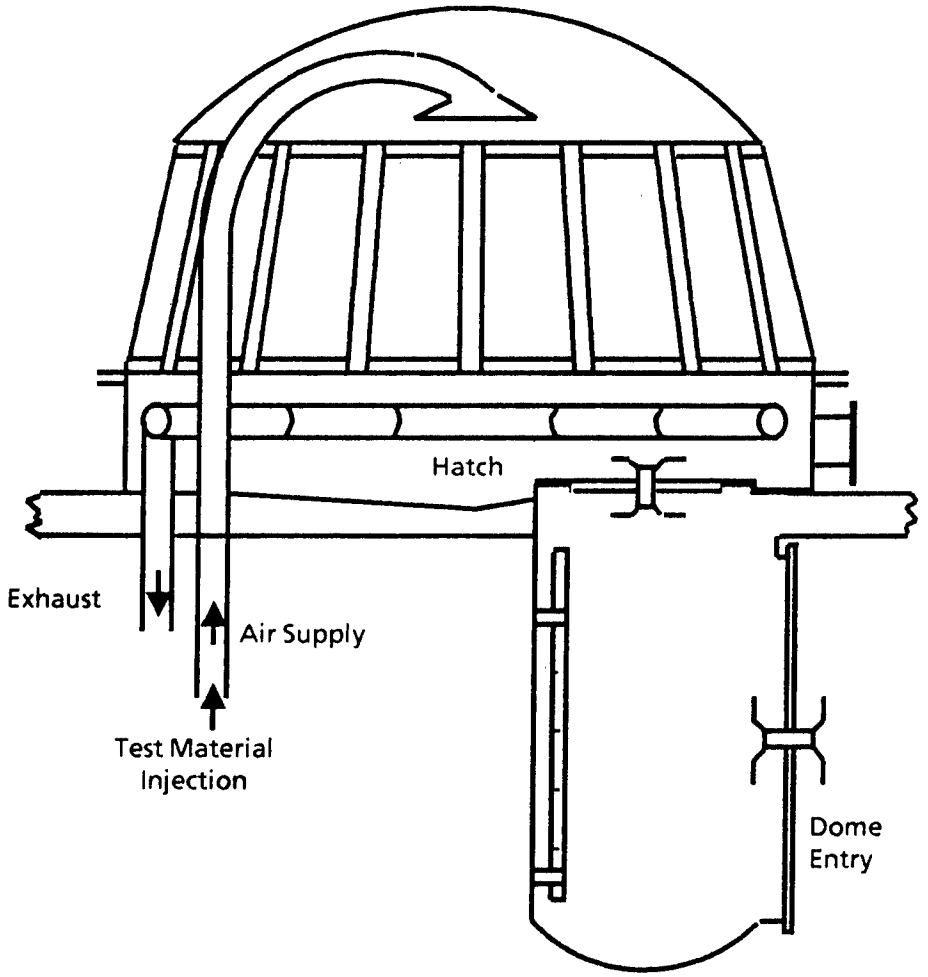


Figure 4.2-1. Cross Section of Thomas Dome.

In order to directly determine the variations in test article concentration from point to point in an exposure chamber, one must measure the concentration of test articles at a number of locations within the exposure chamber. Most efforts to characterize chamber distributions attempt to obtain a "snapshot" of chamber distribution by obtaining as many measurements as possible in a short period of time, and by analyzing the observed variation in test article concentrations (Moss *et al.*, 1982; Griffis *et al.*, 1981; Yeh *et al.*, 1986). As larger chambers are employed, it becomes difficult to measure enough points in a sufficiently short time so that the observed toxicant concentrations can be said to represent the chamber at one time. The 23 m^3 Thomas Domes are difficult to characterize using conventional measurement methods for test-article concentration. Measurements of test

article distribution in other inhalation chambers indicate that, in general, the test article distribution within the chamber depends on both the design of the chamber and the design of the air/test article delivery system (Yeh *et al.*, 1986). For this reason, test article distribution studies should be done as a function of chamber operating conditions, further increasing the measurement burden in these studies. Therefore, we are investigating methods to characterize toxicant distributions in large exposure chambers as part of this measurement effort.

Moss (1985) has suggested a method for reducing the requirements for rapid sampling. Sequential measurements of test article concentration are made from a central reference point in the chamber. These data are a measurement of the variation of the test article concentration with time. Reference-point measurements are interleaved with measurements at different points within the exposure chamber to determine the total (time and spatial) variation of test article concentration. Statistical analysis is used to determine the spatial variation of the test article concentration (Sokal and Rohlf, 1969). This method appears to work as long as the variation of test article with time is not greater than the spatial variation within the exposure chamber.

In order to apply spatial distribution measurements to the Thomas Domes, we combined the two approaches described above by measuring test article concentration at numerous points and deliberately varying the operating parameters of the dome in a random manner. The measured concentrations were analyzed using factorial analysis of covariance. The results of this analysis provide a measure of spatial variation within the dome and also provide an indication of what operational parameters most strongly influence this variation. The relative importance of the operational parameters can be used to determine what changes or repairs need to be made in the operation of the dome if the measured spatial variation is too large.

METHODS

For vapor-phase test articles, Brownian diffusion is the major mechanism by which the test article can move across airflow patterns. To study distribution characteristics of gaseous materials in the Thomas Domes, two different materials were used in two measurement phases. In phase one, propane, a gas with a low molecular weight and a high diffusion constant, was used. In the second phase, dodecane, a much heavier hydrocarbon with one-half the diffusion constant of propane, is being used.

Test Article Generation

For the first measurement phase, a tank of pure propane equipped with a flash arrestor was connected to a rotameter, which metered the propane into the dome supply air at low concentrations. To ensure thorough mixing, a counterflowing jet was used to introduce the propane into the airflow of the main chamber. Morgan and Brinkworth (1976) demonstrated that a

turbulent jet mixes into a counterflowing turbulent flow in a pipe in less than 10 pipe diameters. The Reynolds numbers for the flows must be above 1,000 for the main flow and 10,000 for the jet. In order to obtain these high Reynolds numbers in the counterflowing jet, propane was mixed with metered dilution air. The mixture enters the dome through the contaminant introduction system, which automatically switches to bypass if there is no airflow through the dome. Figure 4.2-2 illustrates the propane generation system.

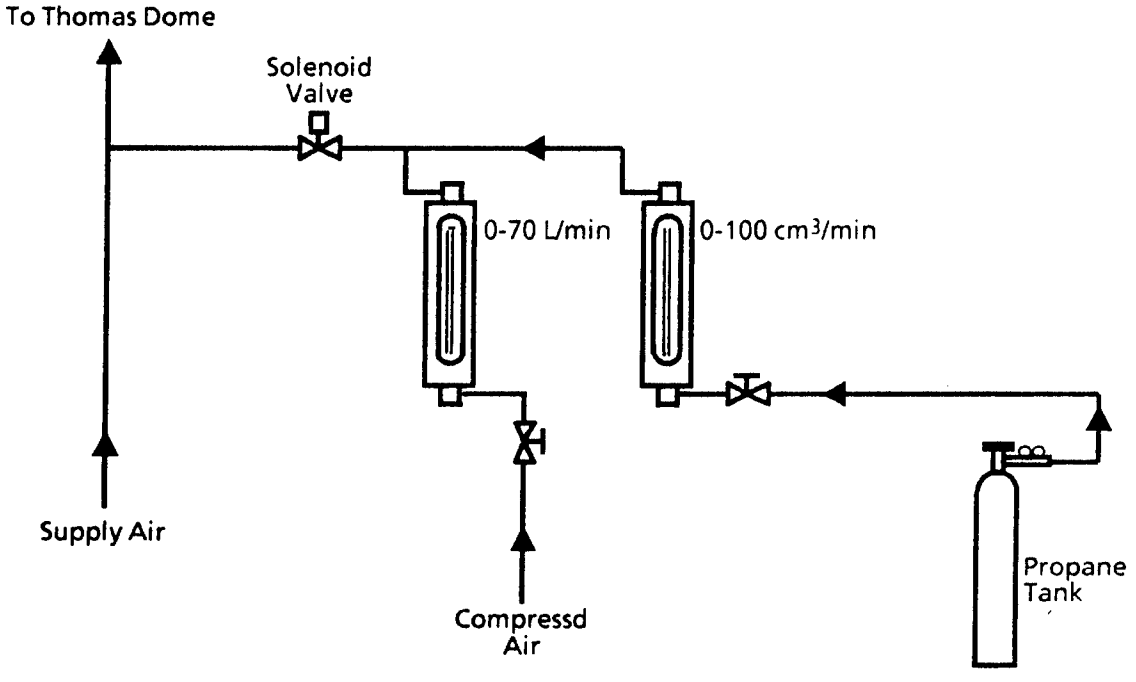


Figure 4.2-2. Test Gas Injection System.

In the generation system for the second measurement phase, dodecane will be metered into a heated evaporation tower. Metered air will flow up through the tower and into the dome contaminant introduction system. The temperature, both upstream and downstream of the tower, will be monitored to prevent overheating. The airflow and flow of the compound into the tower will determine the concentration.

Analytical Method

Hydrocarbon vapors or gases can be detected using a hydrocarbon analyzer based on the flame ionization detector (FID). Six Beckman Model 400 Hydrocarbon Analyzers were used to monitor concentration at various points within the dome. One analyzer monitored concentration at the diffuser ring (inlet) and the exhaust line (outlet). The remaining five analyzers sampled from locations within the dome. Each analyzer was situated between the two windows in each dome. In each of the ten windows, five stainless steel probes, 3/8" OD and 76" long, were placed at five

different levels. These probes were mounted so they could be moved radially to allow sampling from all points in a sampling plane. The sampling locations are shown in Figure 4.2-3. The probes were connected to a manifold system of solenoids controlled by an IBM PC. The manifold outlet was connected to a hydrocarbon analyzer, which had a pump in line before it. The flow rate of the sample through the analyzer was measured with a rotameter on the exhaust side of the instrument. Flow rate was set at 2.0 L/min. All six analyzers were equipped with pop-off valves to prevent damage to the instruments in the event of a sudden surge of pressure. Compressed air, in combination with 100% pure hydrogen, was used as an FID fuel mixture. The analyzers were calibrated with a standard calibration gas of 33 ppm propane. Figure 4.2-4 illustrates the experimental setup. The dome input air was used as baseline for all analyzers.

Data from each analyzer output were collected by using a microcomputer data acquisition system based on the IBM PC computer. This program also operated the electrical valves connecting the analyzers to each probe sequentially. To allow operators to view the data in real time, analyzer output was also connected to strip-chart recorders.

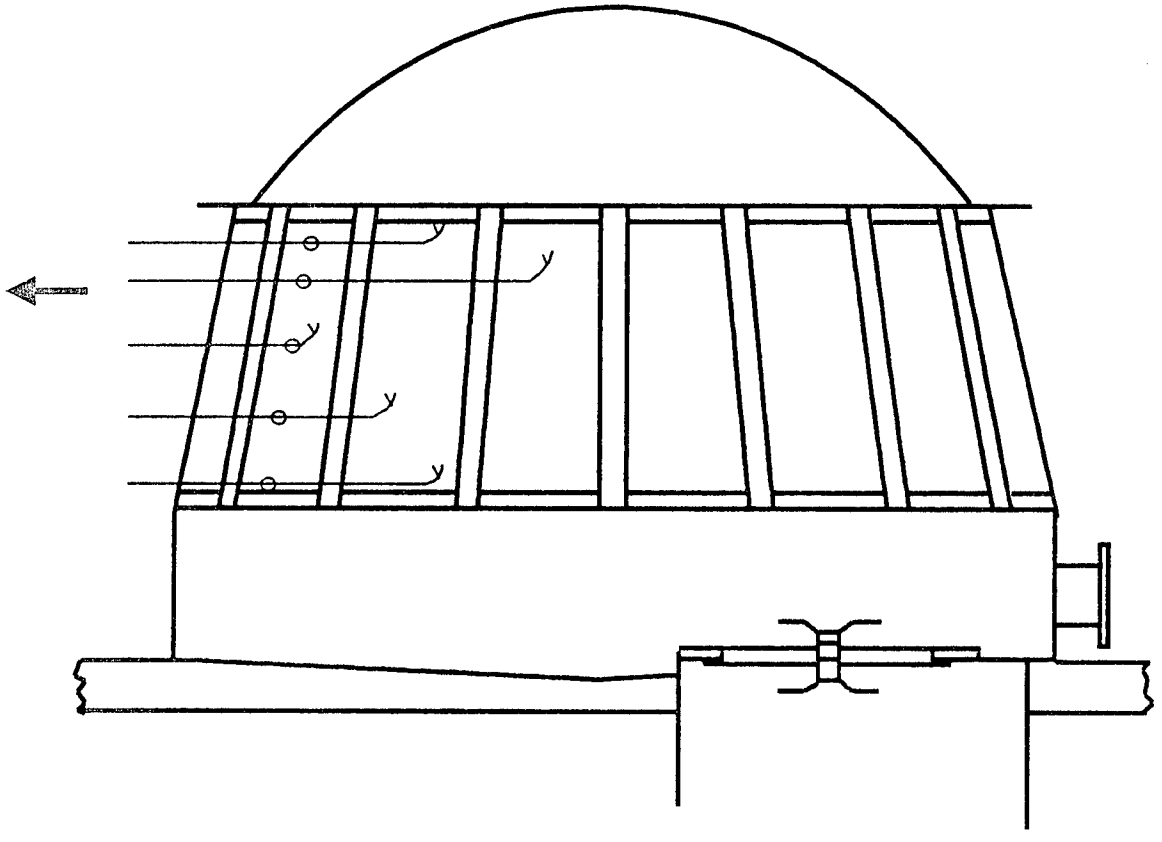


Figure 4.2.3a. Sampling Probe Levels.

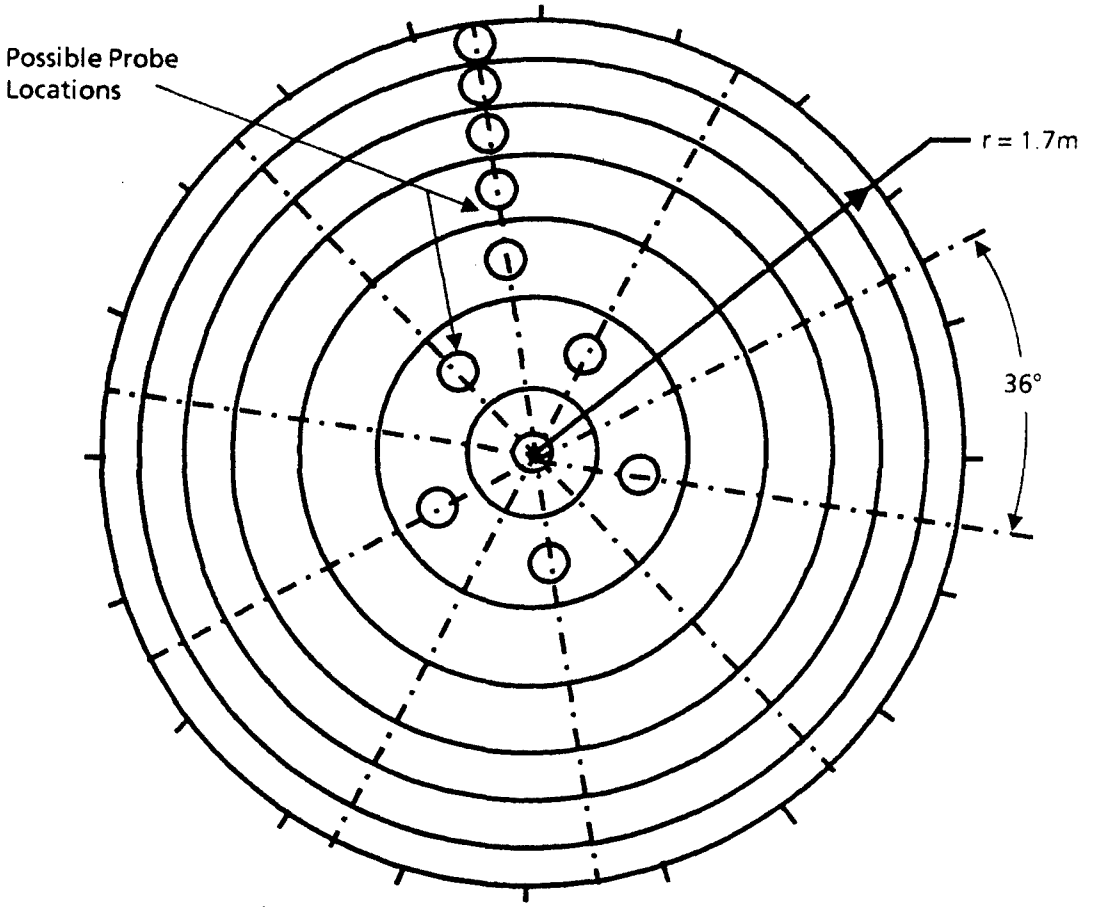


Figure 4.2-3b. Sampling Probe Radial Positions.

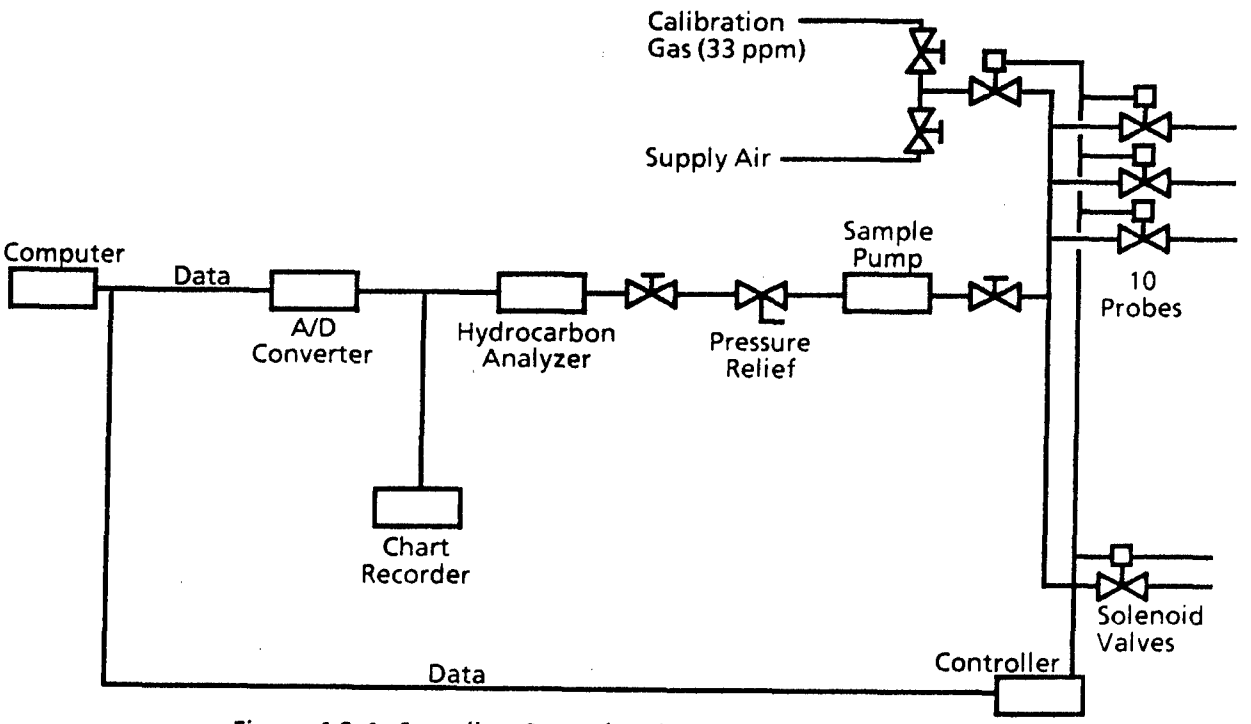


Figure 4.2-4. Sampling Control and Data Acquisition System.

Probe locations were randomly selected at the beginning of each day. The dome was operated at eight different flow rates, ranging from 25 CFM to 95 CFM, and at eight different pressure settings. The combinations of these two parameters were chosen randomly in a manner that ensured that each preselected parameter value was equally represented. For each of the 5 analyzers that sampled the dome volume, data were collected in sets of 10 concentration values from 2 windows. Before and after each data set was collected, a baseline reading was taken from the inlet air supply of the chamber. The sixth analyzer monitored concentration at the inlet and the exhaust and took baseline readings, recording the rise and fall times in the dome. The hydrocarbon analyzers were calibrated prior to distribution measurements using dilutions of the 33-ppm standard gas prepared in Teflon sampling bags. To monitor the drift of each hydrocarbon analyzer, single concentrations of standard gas were measured three times daily. These data were used to determine when an analyzer had drifted outside the calibration confidence interval.

Statistical Methods

To determine the relationship between the concentration of the test article and operating parameters of the dome (flow, pressure, external temperature, internal temperature, analyzer drift, sampling point position, and time), a regression procedure (RS1/FIT MULTIPLE) with concentration as a dependent variable was used. A factorial analysis of covariance was performed to find differences in position and time with covariances of flow, pressure, internal temperature, external temperature, and analyzer drift (BMDP2V). Because position and sample are categorical variables, a log-linear model procedure was done with the above variables and concentration. Normality of concentration was checked using a Kolmogorov-Smirnov test (Sokal and Rohlf, 1969). All of the above-mentioned analyses were required because this analysis was exploratory.

Leak Rate Measurements

The leak rate of the dome was measured by the method of Mokler (9). The pressure in the dome under study was lowered to 2 in. H_2O, and the dome was isolated from the air supply and exhaust system. A standard pressure gauge (Wallace and Tiernan) was used to monitor the decrease in pressure in the dome caused by air leakage into the dome. The time for the pressure to rise by 1 in. H_2O was recorded. From these data, the fractional leak rate of the dome can be calculated.

RESULTS

Dome Leak Rate

Dome leak rates were measured during operation of the dome, but no extraordinary efforts were made to seal small leaks prior to data analysis. Initial leakage rates were high, indicating that excessive air was infiltrating the dome and/or the air delivery system. Investigation revealed that the

manual shut-off valves present in the air supply system did not seat completely. It was also found that cycling the dome pressure served to seat the dome cap. The leak rate of the chamber decreased after pressure in the dome was lowered and returned to ambient a few times. The initial leak rate was 26.4% of the chamber flow rate. After seating the dome cap, the leak rate was found to be 15.3%. The measured equilibrium concentrations of the chamber inlet and outlet differed by 1.3 ppm, or 5.4% of the average propane concentration. Statistical analysis of the data demonstrated that the majority of this difference was correlated with changes in the analyzer baseline. When these effects were mathematically removed, there was no significant difference between inlet and outlet concentrations.

Mixing Characteristics of the Domes

Analysis of the rising propane concentration after introducing the propane flow into the chamber indicated that the Thomas Dome behaves as a well-mixed system for gaseous test articles. Table 4.2-1 shows the calculated and measured rise times obtained during these measurements. The decrease of the propane concentration with time after shutting off the propane flow can be analyzed by the method of Cholette and Cloutier (1959) to yield information concerning the poorly mixed fraction of the chamber volume. Figure 4.2-5 is a plot of these data obtained at a flow rate of 95 CFM. The best least-squares fit to the data resulted in the equation shown in Figure 4.2-5. This equation corresponds to the flow conditions under which 88 to 95% of the chamber is well mixed with the incoming flow and some of the exhaust flow is generated by piston flow of the air within the chamber.

TABLE 4.2-1. COMPARISON OF DOME RISE TIME WITH THEORETICAL RISE TIME FOR PERFECT MIXING

Flow Rate (CFM)	Pressure (mmHg)	Flow/Volume	
		Observed	Calculated
25	10	0.040	0.0354
	18	0.0382	
	24	0.0475	
	36	0.0396	
95	Mean ± SD	0.0413 ± 0.004	
	5	0.153	0.135
	10	0.147	
	18	0.085	
	24	0.156	
	36	0.163	
	Mean ± SD	0.141 ± 0.032	

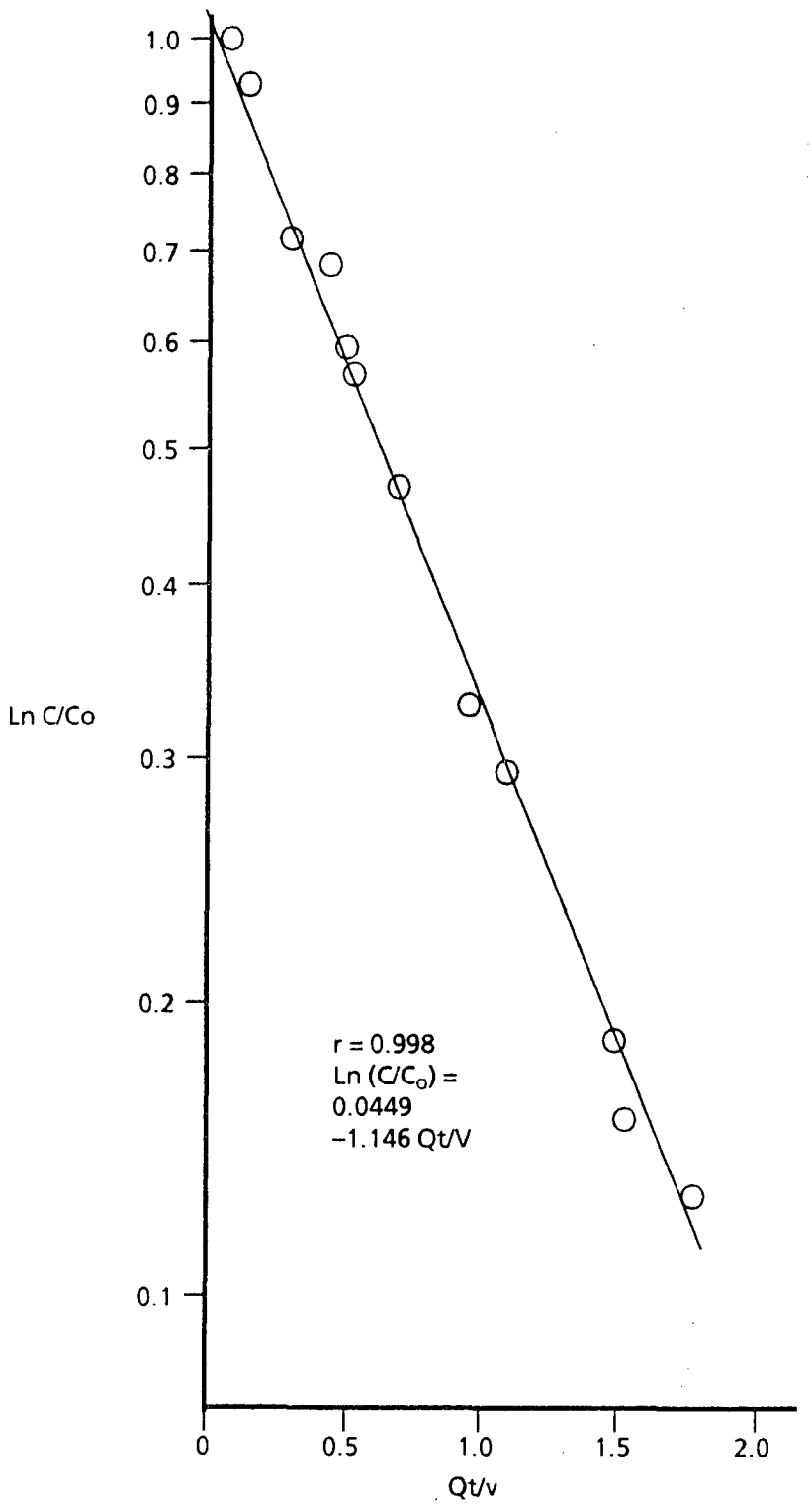


Figure 4.2-5. Graphical Analysis of Dome Mixing Characteristics
Q = Dome flow rate (cubic feet/min)
t = Time (min)
v = Dome volume
C_o = Steady state gas concentration
C = Gas concentration at time t.

Characteristics of the Measurement System

The propane metering system produced an average propane concentration of 23.6 ± 0.96 ppm (mean ± standard deviation) in the dome. The inlet and outlet concentrations showed a 7% fluctuation about the mean. The measured propane concentration showed trends with time, but there was no persistent concentration pattern within the dome itself. Propane distributed uniformly throughout the dome, and the fluctuations about the mean at any point were random. The propane distribution data were not normally distributed by the Kolmogorov-Smirnov normality test (Figure 4.2-6), showing an excessive number of data points that were statistically different from the mean. Regression analysis showed that the temperature at the top of the dome was the most significant factor influencing the concentration, followed by baseline drift of the analyzer and the pressure in the dome (Table 4.2-2). Removing the variability attributable to these parameters reduced the data range by eliminating low analyzer readings (Figure 4.2-7).

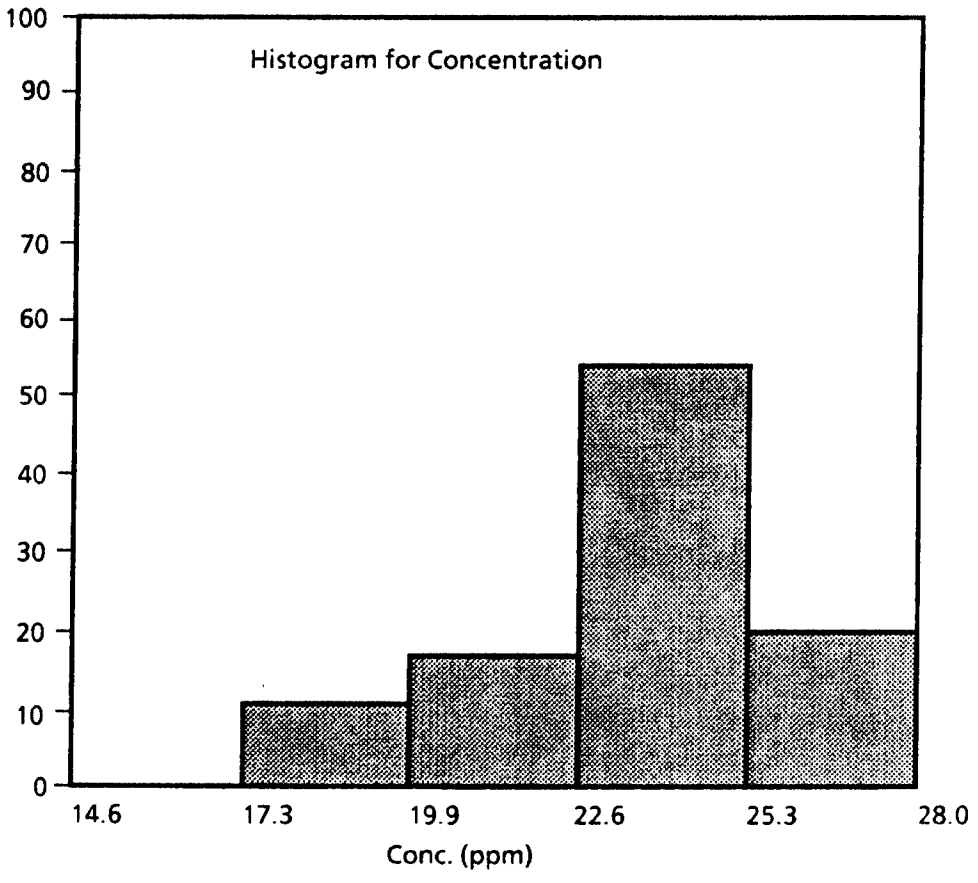


Figure 4.2-6. Distribution of Hydrocarbon Analyzer Measurements.

TABLE 4.2-2 ANALYSIS OF VARIANCE FOR DOME TEST ARTICLE CONCENTRATION DATA

Significant Variable	Multiple R^2
Top Dome Temperature	31%
Analyzer Baseline	20%
Dome Pressure	12%

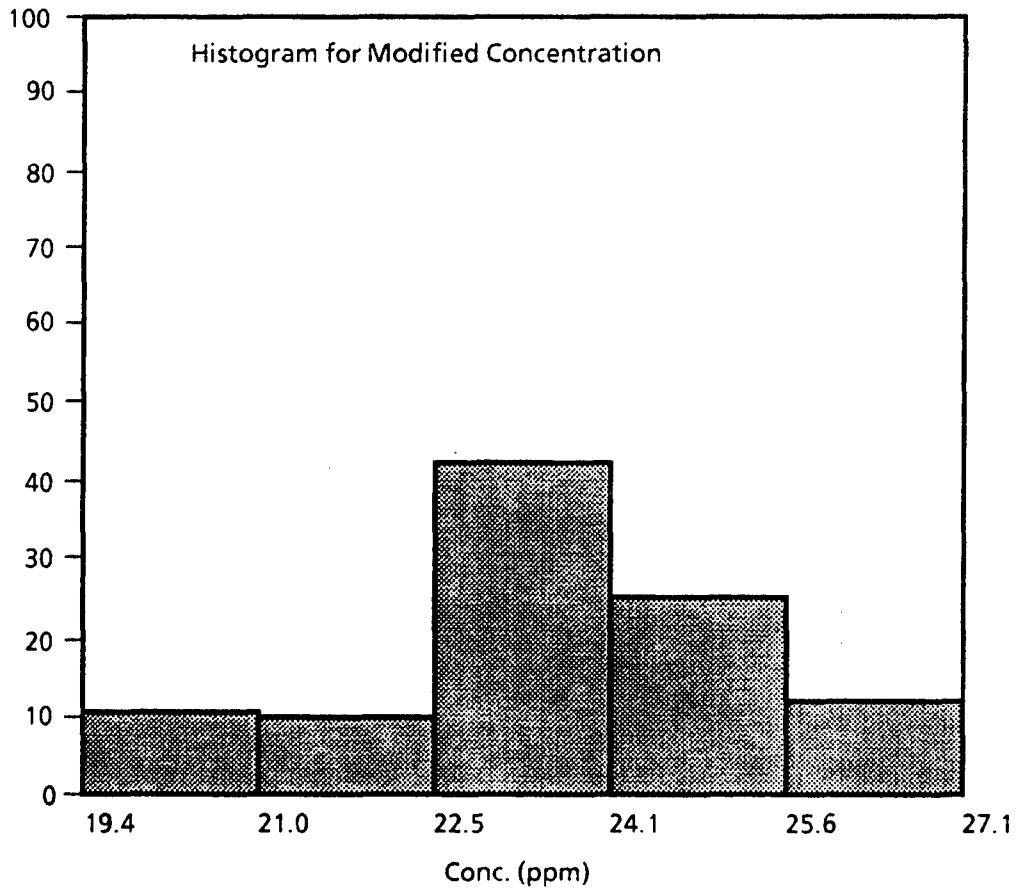


Figure 4.2-7. Distribution of Calculated Hydrocarbon Analyzer Concentration Measurements after Removing Explained Variability.

The hydrocarbon analyzers exhibited good short-term stability. However, their calibration shifted by detectable amounts from day to day. Calibration curves were developed each day to assess the stability of the analyzers. The parameters from a least-squares analysis of the daily calibration data are shown in Table 4.2-3. The individual daily calibration curves for each analyzer were used to convert the analyzer voltage levels to parts per million of propane.

TABLE 4.2-3 HYDROCARBON ANALYZER CALIBRATION DATA

Analyzer					
1		2		3	
Slope	Intercept	Slope	Intercept	Slope	Intercept
2.42	0.434	2.01	0.520	2.01	-0.191
2.43	-0.088	2.02	-0.039	2.08	-0.452
2.43	-0.088	2.02	-0.039	1.99	-0.610
4		5		6	
Slope	Intercept	Slope	Intercept	Slope	Intercept
2.06	0.0273	2.03	1.06	2.01	-0.088
2.08	-0.453	1.82	0.744	1.95	-0.793
2.08	-0.453	1.82	0.744	1.95	-0.793

Spatial Distribution in the Dome

The individual propane concentration measurements were compiled and analyzed using factorial analysis of covariance. Statistical tests were applied under the assumption that the data were normally distributed. The relative standard deviation of the measured propane concentration was 9%. When the effects of operational parameters were removed, the relative standard deviation of the spatial distribution in the dome was 6.4%. This value is taken to be the inherent spatial variation of the Thomas Domes when operated with a light, gaseous test agent. Changes in operating parameters accounted for the remaining variation in the measured concentrations. The factors that affected test article distribution were, in decreasing order of effect, local temperature fluctuations, analyzer initial zero, and pressure.

DISCUSSION

When the Thomas Dome was operated at 1 mmHg below ambient pressure, there were no noticeable leaks; but, as pressure was decreased, leaks did become detectable. The average overall leak rate calculated from the inlet and outlet concentration data was 5.4%, less than the 15.3% observed during leak measurements. Because the apparent difference between dome inlet and outlet concentrations correlates with changes in analyzer baseline, we assume that the measured leakage was due to the inability to completely close the manual inlet and exhaust valves.

The Thomas Domes appear to behave in a manner described by a well-stirred tank model, indicating that for light hydrocarbons such as propane, the test article flow at the inlet is mixed into the entire chamber volume with little loss or delay. The fraction of the volume in the dome that mixed poorly with the rest of the chamber (dead volume) was between 5 and 12% of the total

volume in the dome. No shunting of material from the inlet to the outlet of the chamber was observed, which indicates that the material supplied to the chamber reaches the animals without significant loss. Some of the airflow in the chamber appeared to exit the chamber by displacement by the incoming air, as if the incoming air behaved like a piston. Cholette and Cloutier (1959) showed that the time required for the outlet concentration to fall is the time necessary to displace the unmixed volume of the chamber. This displacement must arise from the piston-like component of the inlet flow because there is no exchange of material between the dead volume and the active volume of the chamber. Therefore, the fraction of the inlet flow that acts like a piston can be calculated from the flow rate, time for displacement, and size of the dead volume. For the Thomas Domes, 5.3% of the inlet flow acts like a piston.

The spatial distribution for light hydrocarbons in the Thomas Dome varies by only 6.4%, which suggests that very uniform doses are delivered to animals exposed in the chambers. Analyzer stability remained a statistically significant contributor to observed data variation. Therefore, even with statistical control of analyzer calibration, measurement technique remains an important determinant of the experimenter's perception of test article distribution. The variation of spatial distribution in the dome is sufficiently low that a major factor in its measurement is the inherent drift and inaccuracy of the measuring method. Dome pressure was also a significant contributor to the variability of test article distribution, indicating that this operational parameter must be statistically controlled by a covariant experimental design if it cannot be stabilized.

The ability to measure small percentage variations in chamber concentration depends critically on the overall stability of the analytical system used. Analysis of the hydrocarbon analyzer voltages using a single calibration curve suggests that the measured propane distribution in the dome is low in one region, possibly as the result of leaks. This region of low concentration involves two of the six hydrocarbon analyzers. However, when the analyzers are calibrated daily and the measured propane concentrations are computed using these calibrations, no persistent patterns of low or high concentration are found.

These statements apply to operation of the Thomas Domes with gaseous test articles capable of rapid diffusion across airflow paths. Future measurements will indicate the effects of using materials of low diffusion capacity, such as heavy vapors and aerosol particles, on dome distribution and operation.

REFERENCES

Cholette, A. and L. Cloutier. 1959. Mixing efficiency determinations for continuous flow systems. *Can. J. Chem. Eng.* XX:105-112.

Griffis, L.C., R.K. Wolff, R.L. Beethe, C.H. Hobbs, and R.O. McClellan. 1981. Evaluation of a multitiered inhalation exposure chamber. *FAT* 1:8-12.

Heminway, D.R., D. Sylvester, P.N. Gale, P. Vacek, and J.N. Evans. 1983. Effectiveness of animal rotation in achieving uniform dust exposures and lung dust deposition in horizontal flow chambers. *Am. Ind. Hyg. Assoc. J.* 44:655-658.

Heminway, D.R., R.L. Carpenter, and O.W. Moss. 1982. Inhalation toxicology chamber performance: A quantitative model. *Am. Ind. Hyg. Assoc. J.* 43:120-127.

Mokler, B. V. and R. K. White. 1983. Quantitative standard for exposure chamber integrity. *Am. Ind. Hyg. Assoc. J.* 44:292-295.

Morgan, W.D. and B.J. Brinkworth. 1976. Upstream penetration of and enclosed counterflowing jet *Ind. Eng. Chem. Fundam.* 15:125-127.

Moss. O.R., J.R. Decker, and W.C. Cannon. 1982. Aerosol mixing in an animal exposure chamber having three levels of caging with excreta pans. *Am. Ind. Hyg. Assoc. J.* 43:244-249.

Moss, O.R. 1985. Personal communication.

Sokal, R.R. and F.J. Rohlf. 1969. *Biometry.* San Francisco: Freeman & Company.

Yeh, H.C., G.J. Newton, E.B. Barr, R.L. Carpenter, and C.H. Hobbs. 1986. Studies of the temporal and spatial distribution of aerosols in multi-tiered inhalation exposure chambers. *Am. Ind. Hyg. Assoc. J.* 47:540-545.

4.3 MODIFICATION OF THE NMRI/TD INHALATION EXPOSURE FACILITY AT WPAFB

E.C. Kimmel, V.L. Harris, and D.W. Winsett

INTRODUCTION

Investigation of the potential inhalation hazards posed by materials in use or proposed for use in Naval operations constitutes a significant portion of the THRU research effort. In previous years inhalation studies required to satisfy THRU research objectives have been conducted in AAMRL facilities; however, with the advent of AAMRL facility modifications, an alternative exposure capability was required to accommodate more urgent research needs. Consequently, an agreement was reached between the THRU and NMRI/TD to refurbish, on a limited scale, a portion of NMRI/TD's inhalation exposure laboratory.

In cooperation with Naval personnel, the THRU Exposure Engineering and Operations staff performed a comprehensive evaluation of the NMRI/TD exposure facility to determine the extent of modifications necessary and feasible to render the facility operational and in compliance with conventional standards and regulatory guidelines. This was necessary primarily because the system's existing configuration was not well suited for use with the materials currently of interest to the Navy. As a result of this evaluation, a program was implemented to restore two of the four Rochester-type (Leach *et al.*, 1959) inhalation exposure chambers to full operational status as dynamic exposure systems.

Exposure System Modifications

The exposure system restoration program was implemented in several stages designed to impose minimal interference with other ongoing Navy research projects.

Exposure Chamber Reconditioning

The integrity of the exposure chamber was assessed using methods similar to those described by Mokler and White (1983) in which the rate of pressure change over time is measured in exposure chambers under static conditions. This quantitative estimate of the relative leak rate of the chambers indicated that remedial action was required to bring chamber integrity up to minimum acceptable standards for inhalation exposure studies. The most obvious deficiencies in the chambers were the lack of adequate seals around windows and doors. New gasket material was installed using up-to-date techniques for sealing the gaskets. All fractured and penetrated flat surfaces were replaced. Additional reconditioning included repair and modification of door latches. Subsequent quantitative assessment of chamber integrity illustrated a need for additional leak abatement at

structural butts and joints. These leak problems were resolved by applying semipermanent, inert sealing compounds to the welds.

Exposure System Air Movement and Control Systems

To meet modern exposure chamber airflow and pressure requirements for conducting animal exposures, several modifications were made to the chamber's inlet and exhaust air supply and control systems. Satisfying the number of air exchanges per hour recommended by the 1982 EPA Health Effects Test Guidelines (U.S. EPA, 1982) for animal exposure studies required connecting the chamber's air supply lines to a higher capacity blower unit. This was accomplished by reconnecting the chamber's exhaust lines to an existing blower that served a chemical fume hood in an adjunct laboratory. Careful measurements of the capacity of air movement in the blower demonstrated that this unit was fully capable of supplying the needs of both the fume hood and the exposure chambers under conditions of full demand. Air filtration devices in the chamber inlets were changed, providing greater filtration capacity to accommodate the higher operating chamber flows and more refined filtration capability. The existing devices for measuring flow were recalibrated using calibration instruments traceable to NBS standards; these existing devices, though accurate, were of limited sensitivity at all allowable chamber operational flows. Hence, additional flow monitors were installed to expand the characterizable operating scale of the chambers. Similar problems were encountered with operating devices to measure pressure in the chambers. Consequently, these devices were likewise replaced with expanded scale, increased sensitivity instruments. Airflow and pressure control instrumentation also received critical assessment, and modifications were found to be necessary. Nonfunctional exhaust duct pressure/flow dampers were repaired, and inlet airflow control valves were installed so that the appropriate chamber versus environmental pressure differentials could be maintained at all operational flow rates. Additional levels of pressure and flow control in the chamber were provided by the installation of electronically actuated isolation valves; the primary function of these valves was to isolate the chamber from the environment in the event of an accidental loss of power to the blower unit. In addition to improvements in the airflow monitoring and control systems, pressure loss measurements of duct work integrity led to some minor repairs of the existing air ducts between the exhaust port of the chamber and the previously existing blower port located on the roof of the NMRI/TD research facility.

Chamber Exhaust (Air Effluent) Cleansing System

The most extensive and critical renovation of the exposure system was the connection of the chamber's exhaust system to an effluent scrubber. A network of new duct work was installed from the exposure chamber's previous blower exhaust port to the water-misting scrubber unit downstream from the chemical fume hood described earlier. With the combined effluent scrubbing

demands placed upon this system, it was necessary to make modifications in the scrubber unit. These modifications consisted of the installation of a water-misting nozzle with a higher capacity/finer droplet size, accompanied by the requisite increase in capacity of the water supply line, delivery controls, and drainage capability. In addition, a controllable water usage monitor plus backup systems were installed.

Nonmechanical Exposure System Modifications

An integral part of the exposure system restoration and reactivation program included implementing system operational procedures and operational safety programs. A record-keeping system for calibration, system modification, usage, repair, preventive maintenance, and safety control was instituted. Moreover, these programs were instituted in complete coordination with Naval programs of similar nature.

SUMMARY

Once the exposure system renovation program was completed, a systems-testing program was instituted in which simulated exposures using an innocuous/surrogate toxin were conducted. An analysis of the newly renovated system's capabilities was performed by both THRU and NMRI/TD personnel. All system components were deemed acceptable for use in inhalation studies limited by the following criteria.

1. Only relatively nontoxic substances should be tested in this system in adherence to definitions proposed by Klaassen and Doull (1980): materials expected to be categorized as moderately toxic (0.5 to 5.0 g/kg or one oz. to one pint probable lethal dose to humans or less).

2. Materials to be tested should have low vapor pressure and high water solubility/miscibility. These criteria were imposed primarily because of limitations beyond immediate THRU and NMRI/TD control, such as the need for secondary containment structures. Subsequently, the renovated, reactivated NMRI/TD inhalation exposure facility was used to conduct studies to evaluate the inhalation toxicity of four materials proposed as hydraulic fluids for Naval systems.

REFERENCES

Leach, L.J., L.J. Spiegl, R.H. Wilson, G.E. Sylvester, and K.E. Lauterbach. 1959. A multiple chamber exposure unit designed for chronic inhalation studies. *Am. Ind. Hyg. Assoc. J.* 20:13.

Klaassen C.D. and J. Doull. 1980. Evaluation of safety: Toxicologic evaluation. In: J. Doull, C.D. Klaassen and M.O. Amdur, eds., *Toxicology* 2nd Edition, pp. a4-27. New York City: MacMillan Publishing Co.

Mokler, B.V. and R.K. White. 1983. A quantitative standard for exposure chamber integrity *Am. Ind. Hyg. Assoc. J.* 44(4):292.

U.S. Environmental Protection Agency. 1982. Health Effects Test Guidelines. EPA 560/6-82-001, p. 432. Office of Pesticides and Toxic Substances, Office of Toxic Substances, (TS-792). Washington, DC: U.S. Environmental Protection Agency.

4.4 THE THRU WHOLE-BODY INHALATION EXPOSURE CHAMBER

E. C. Kimmel and R. L. Carpenter

INTRODUCTION

Part of the program to expand and upgrade AAMRL/TH inhalation exposure facilities included the design and fabrication of new small-animal whole-body exposure chambers. The new THRU chamber offers distinct advantages compared to the Rochester- and Longley-style chambers previously in existence at AAMRL/TH. The chamber design incorporates many of the recent advancements in inhalation chamber technology (Drew, 1978), which were not available to pioneer exposure chamber designers. Principal design objectives were to satisfy well-known, scientific and traditional performance criteria for whole-body inhalation exposure chambers, such as providing a stable, well-controlled, and readily characterizable toxicant delivery in concert with fulfilling regulatory requirements and suggestions for housing and maintaining laboratory animals. Additional design objectives focused on satisfying economically practical criteria. In a comprehensive analysis of exposure facility design, Carpenter and Beethe (1980) reported that the typical construction and operating costs of the inhalation exposure laboratory were nearly twice that of comparable research laboratory facilities. Also, inhalation laboratories require three times as much floor space as other laboratories. The THRU chamber was designed to be suitable for a variety of exposure regimens from short-term limit test exposures of relatively few small animals (e.g., 10 rats) to long-term chronic exposures of several small animals (e.g., 32-48 rats). Features of the THRU chamber make it adaptable for conducting exposures to test materials existing in a variety of physiochemical forms, including relatively large aerosol particles and droplets as well as vapors and gases or combinations of both.

Design Objectives

The THRU chamber is a slightly larger and highly modified version of the 27-in. inhalation exposure chamber discussed by Hinners *et al.* (Hinners, 1968). Overall chamber dimensions can be found in Figure 4.4-1. Basically, the THRU exposure chamber has a cuboidal exposure volume of 500 L with pyramidal inlet and exhaust plenums that bring the total chamber volume to 690 L. The inlet plenum geometry was designed to optimize uniform distribution of aerosol toxicants in the exposure volume and minimize rotational air (hence toxicant) flow. This modification to the Hinners chamber, as well as the development and inclusion of a distributive exhaust manifold (not shown), was based on the work of Carpenter and Beethe (1980). These modifications were specifically directed toward addressing problems with rotational and recirculatory air currents within the chamber exposure volume at recommended chamber operating flows and pressures. Elimination of

these local flow phenomena within the exposure volume facilitates uniform toxicant distribution in the chamber and mitigates the problem of animal exposure to off-gassing animal excreta by-products. This problem is caused by refluxing airflow across animal waste collection surfaces to the animal breathing zone.

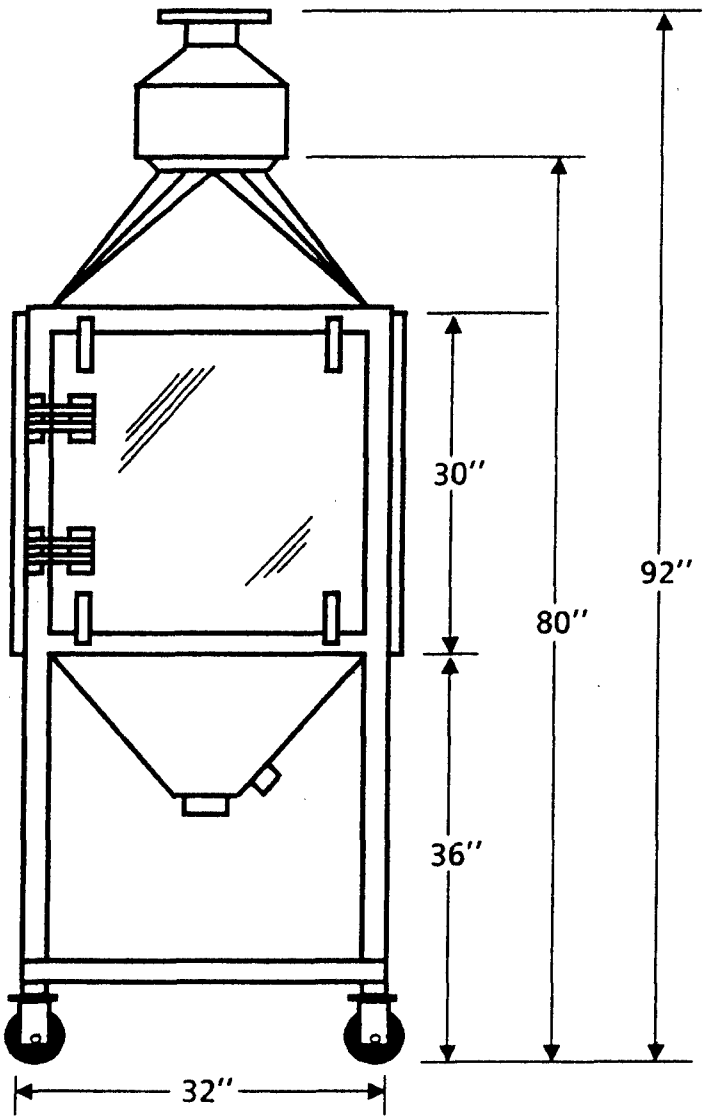


Figure 4.4-1. Dimensions of THRU Inhalation Exposure Chamber.

Another chamber modification developed by Carpenter and Beethe (1980) was the use of the opposed normal versus tangential air inlet configuration. This configuration provides suitable toxicant mixing while abating rotational airflow in the chamber. The THRU chamber utilizes a second generation step of this development, incorporating an annular orifice inlet configuration for the air (toxicant) delivery into the design. The annular orifice provides a continuous circular opposing jet for toxicant mixing instead of two jets as with the opposed-normal configuration.

Furthermore, the orifice size can be adjusted to vary air flow velocity. Hence, the inlet airflow can be "customized," if necessary, to suit toxicants with unusual physiochemical characteristics.

Inhalation chamber design features similar to those of Moss *et al.* (1982), which address multitiered animal housing, have been included in the THRU chamber. In the past, catch pans for animal excreta have been an obstacle to uniform flow and toxicant delivery in exposure chambers; thus, single-tiered exposure configurations were recommended for most aerosol studies. However, available exposure volume was underutilized because placing animals in a single-tiered conformation usually meant that far less than the acceptable 5% of exposure volume occupation by animals was in effect. The THRU chamber is slightly larger than the original 27-in. Hinners chamber to allow for the insertion of a vertical distributive exhaust manifold, passing up through the center of the exposure volume. Exhausting the chamber air in this manner will allow animals to be placed in a multitiered conformation (up to three tiers) in the THRU chamber, thereby increasing the capacity for animal exposure, when necessary, without significantly changing the uniformity of toxicant distribution within the exposure volume.

The vertical and regular horizontal exhaust manifolds are interchangeable and removable, which allows for versatile chamber configuration.

DISCUSSION

In addition to having the aforementioned design features, the THRU chamber is constructed of materials resistant to corrosion. The chamber also includes conventional facilities for prolonged animal housing and ease of animal maintenance. The size of the THRU chamber makes it useful for a variety of exposure regimens. The exposure volume is small enough to allow limit tests at high toxicant concentrations with difficult-to-generate test materials, yet large enough to provide adequate housing for the number of small animals required for most long-term chronic exposure studies. Special attention was given to the design of the animal observation windows and door of the THRU chamber. We were able to include large windows in the design without loss of rigidity or sealing integrity of the chamber.

Other significant design features include: (1) multiple access ports situated to provide ample air quality sampling in the animal breathing zone and minimal obstruction of animal observation windows; (2) rapid disconnect fittings for adjunct air movement ducts without loss of absolute leak sealing at these joints; (3) an automated, high volume, self-contained decontamination system; (4) a unique door-hinge and latch arrangement that minimizes nonuniform gasket compression; and (5) ample auxiliary space on the chamber support structure that allows convenient mounting of chamber flow, pressure, humidity, and temperature monitoring and control instrumentation, without significant increase in laboratory floor space dedicated to the exposure unit.

REFERENCES

Carpenter, R.L. and R.L. Beethe. 1980. Airflow and aerosol distribution in animal exposure facilities in generation of aerosols and facilities for exposure experiments. In: K. Willeke, ed. *Generation of Aerosols and Facilities for Exposure Experiments,* pp. 459-474. Ann Arbor, MI: Ann Arbor Science Publishers, Inc.

Drew, R.T. 1978. Development of the standard inhalation chamber. In: R.T. Drew, ed. *Proceedings of the Workshop on Inhalation Chamber Technology,* pp. 3-9, Upton, NY: Brookhaven National Laboratories.

Hinners, R.G., J.K. Burkhart, and C.L. Punte. 1968. Animal inhalation exposure chambers. *Arch. Environ. Health* 16:194-206.

Moss, O.R., J.R. Decker, and W.C. Cannon. 1982. Aerosol mixing in an animal exposure chamber having three levels of caging with excreta pans. *Am. Ind. Hyg. Assoc. J.* 43:244-249.

4.5 CURRENT THRU ACUTE INHALATION EXPOSURE FACILITIES

J.M. Adams, C.R. Doarn, and E.C. Kimmel

INTRODUCTION

During the past year, modification of the general toxicology laboratory (ambient lab) in Building 79 has necessitated the development of an auxiliary inhalation exposure capability. This system consists of two Wahmann 200-L inhalation exposure chambers on loan from NMRI/TD placed inside one of the Thomas Domes, which serves as a secondary containment unit during inhalation toxicity studies. The design of this exposure system incorporates several modifications to these chambers' usual operational configuration that permit the chambers to be used for a variety of different exposure paradigms. To date, however, they have been used primarily to conduct studies in which the toxicant was in aerosol form.

SYSTEM DESIGN CHARACTERISTICS

The exposure system was constructed as a dynamic airflow system capable of performing as both a passive, free-air (or on-demand) inlet air supply system or as an active, forced inlet air supply system. In either configuration, flow/pressure control valves were placed so that fine regulation and adjustment of inlet airflow and relative pressure in the chamber could readily be effected. Although the nominal size of these chambers is conventionally listed as 200 L (which corresponds to the effective exposure volume), the total chamber volume is 250 L including the inlet and exhaust plenums. Thus, system operational flows were established to be from 1.5 to 2.2 SCFM, corresponding to individual chamber air exchange rates ranging from 12 to 15 exchanges per h. These operational flows were measured as the pressure differential across a 0.325-in. diameter, tapered-edge orifice plate placed in each chamber's exhaust line. Initially, these orifice plates were calibrated with a secondary-flow calibration standard prior to the use of the system. Subsequent calibration with a primary calibration source traceable to NBS confirmed original calibration data. Exhaust airflow in the exposure chamber was provided by a blower unit upstream of a vertical, circulating water stack scrubber. A flow-regulating valve was placed in each chamber's exhaust lines. Regulating both inlet and exhaust airflow allows operation of the chamber at constant pressure over a wide range of supply and exhaust pressures. These adjustments are necessary to maintain balanced chamber operation while using high-flow sampling devices. Absolute filters were placed in both the inlet and exhaust air lines. In addition to having regulating valves for inlet and exhaust airflow, the system was configured with electronically actuated shut-off valves to isolate the exposure chambers in case of power loss to the exhaust blower and forced inlet air supply. This eliminates the potential for accidental contamination of the laboratory area in an emergency situation. These emergency

isolation valves were provided with manual reactivation switches so that chamber flow could not be reestablished without deliberation.

The exposure chambers were located inside of a Thomas Dome, which served as a secondary containment unit. The dome's exhaust system was set to operate at low flow during exposure periods to contend with the event of a contaminant spill. The dome's fire extinguishing system was also operable in the event of a fire. A portion of the dome was set aside as an area for animal housing should it be necessary to keep experimental animals in a contained environment between exposure periods. Although the Thomas Domes were not designed for this purpose, they do provide secondary containment/enclosure features when they are not required as primary exposure chambers. These features include isolation units for housing the toxicant generator and convenient facilities for animal and chamber cleaning.

USE OF THE EXPOSURE SYSTEM

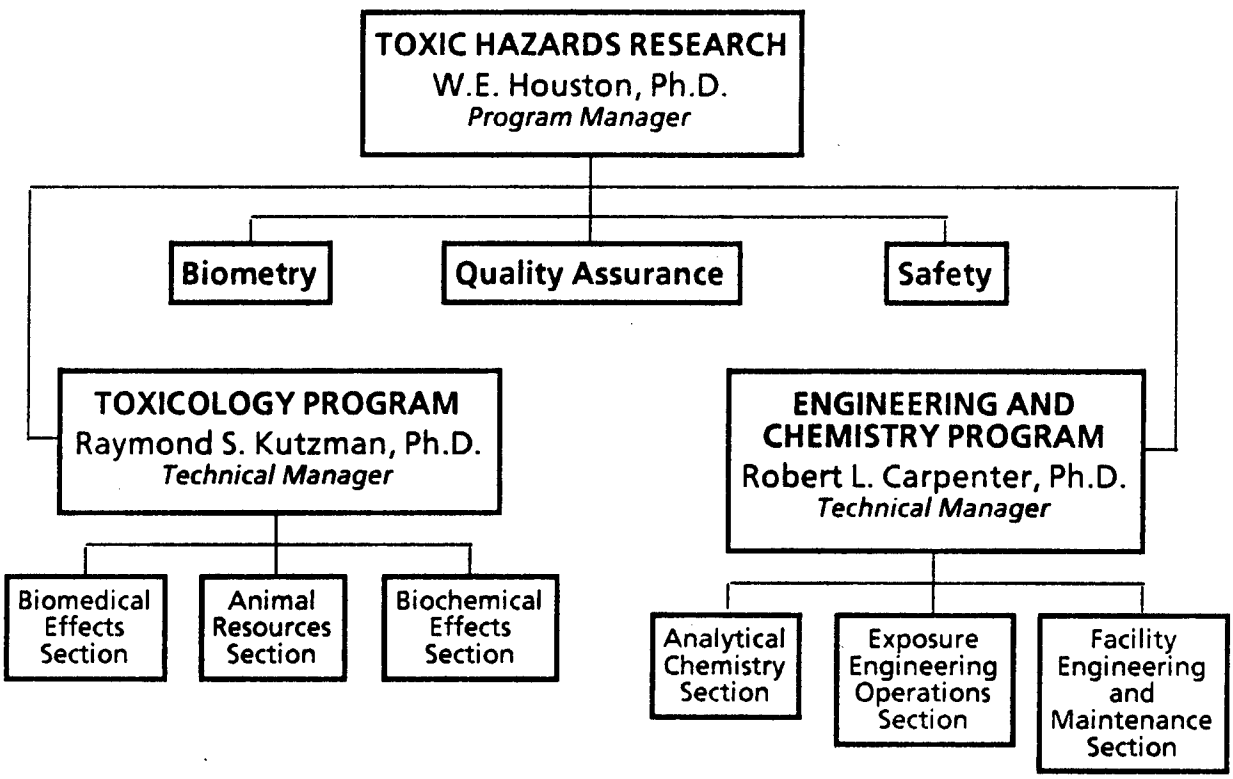
Once the exposure system was set up and operations checks were completed, the system was used to conduct two short-term, acute inhalation exposure experiments. A limit test was conducted by exposing rats to 5.0 mg/L of CPFB. A limit test plus LC_{50} determinations of polyalphaolefin hydraulic fluid toxicity were also conducted. Due primarily to the small exposure volume of the Wahmann chambers, this system is not well suited for the conduct of chronic or subchronic inhalation exposures; hence, system use will be limited to limit-test and LC_{50} determinations.

SECTION 5

APPENDICES

APPENDIX A

NSI/THRU ORGANIZATIONAL CHART



PERSONNEL LIST

NORTHROP SERVICES, INC. – ENVIRONMENTAL SCIENCES

TOXIC HAZARDS RESEARCH UNIT

OFFICE OF DIRECTOR

W.E. (Ed) Houston, Ph.D.
Program Manager

Staff:

Bohanon, Cathy L.
Gaston, Daphne L.

ADMINISTRATION

Lois A. Doncaster
Supervisor

Staff:

Angell, Mary Ann
Branson, Kimberly A.
Kinney, Lowell E.
Ruble, Linda D.

BIOMETRY

Flemming, Carlyle D.
Senior Statistician

Staff:

Surber, Patricia T.

ENGINEERING & CHEMISTRY

Robert L. Carpenter, Ph.D.
Manager

Staff:

Adams, J. Mark
Brade, Donald W.
Brewer, John A.
Doarn, Charles R.
Flowers, Rodney L.
Harris, Vicki L.
Higman, Howard C.
Kimmel, Edgar C., Ph.D.
Leahy, Harold F.
Pollard, Daniel L.
Smutak, Donald A.
Soloman, Kenneth G.
Sonntag, William B.
Stokes, James S.
Szotak, Phyllis S.
Wilson, Richard L.

QUALITY ASSURANCE

Mathias G. Schneider, Jr.
Coordinator

Staff:

Godfrey, Susan M.

TOXICOLOGY

Raymond S. Kutzman, Ph.D.
Manager, Deputy Director

Staff:

Adams, Susan N.
Auten, Kenneth L.
Bailey, Therlo C., Sr.
Barwick, Darwynn L.
Blackford, Robert K.
Brown, Douglas M.
Clendenin, Timothy L.
Conolly, Rory B., Sc.D.
Cook, David K.
Cramer, Jean L., Ph.D.
Culpepper, Brenda T.
Deiser, Patricia A.
Del Raso, Nicholas J.
Dille, Susan E.
Douthwaite, Martha S.
Drerup, Joanne M.
Godfrey, Richard J.
Helton, C. Douglas
Henry, Sandra S.
King, Greg A.
Kinkead, Edwin R.
Malcomb, Willie J.
Mason, Susan K.
McDonald, Gayle A.
Neely, Gloria A.
Nicholson, Jerry W.
Puslat, Karen M.
Reed, Yvonne M.
Ruppert, R. Michael
Schimmel, Brenda D.
Smith, Jessie L.
Vinegar, Allen, Ph.D.
Wagner, Sharon L.
Williams, Deborah L.
Winsett, Darrell W.

APPENDIX B

QUALITY ASSURANCE

Research programs at the Toxic Hazards Research Unit (THRU) are designed for compliance with the U.S. Environmental Protection Agency's (EPA) 40 CFR, Part 792 "Toxic Substances Control; Good Laboratory Practice (GLP) Standards." Protocol production and study supervision and execution also adhere to EPA 40 CFR, Part 798 "Toxic Substances Control Act, Health Effects Testing Guidelines."

Development of a THRU-wide GLP program involved (1) preparation of protocols for the development and writing of Standard Operating Procedures (SOPs), and (2) establishment of a training and education program in GLPs for laboratory personnel. Coordination of SOP production is managed within the Quality Assurance office. Personnel from the Toxicology Program and the Engineering and Chemistry Program have received in-house training on the principles of GLPs, and the senior and supervisory staff participated in a three-day course, sponsored by the USAF and given by the Center for Professional Advancement, on GLP principles as they apply to the Food and Drug Administration.

GLP development at the THRU was initiated by a review of the facilities organization structure and operations. This resulted in focusing activities in the following areas in which special attention is being directed in order to achieve compliance with EPA GLP standards.

GLP Section 792.33. Study Director

Research efforts at the THRU are conducted under technical directives. Technical managers of each technical directive exercise control over all resources (personnel, facilities, and equipment) necessary for the study. These activities are conducted under a projected budget and time element. These resources are utilized by matrix management in order to conduct several studies simultaneously. Shared resources must be clearly identified, and the scope of the technical managers' responsibility and authority must be outlined by laboratory management.

GLP Sections 792.43 and 792.90. Animal Care Facilities and Housing

Because of the shortage of adequate animal holding facilities in the Toxic Hazards Division (Building 79), animals of different species or animals being utilized in different studies must sometimes be housed in the same room. These occurrences are minimized, where possible, and methods used to differentiate animal species, dose groups, and studies are incorporated into each protocol for compliance with GLP standards.

GLP Section 792.47. Facilities for Handling Test and Control Substances

To prevent possible contamination or mix-up of samples, separate areas are needed for the receipt and storage of test substances, and the mixing and storage of mixtures. Because adequate facilities for these purposes do not exist, NSI-ES is attempting to limit the quantity of test substance received to that required to conduct the study, which will significantly reduce the amount of storage space needed. Other measures include designation of test substance mixing areas and storage of those mixtures for ongoing studies.

GLP Section 792.63. Maintenance and Calibrating Equipment

NSI-ES utilizes a centralized U.S. Air Force contract for calibration of equipment utilized in THRU studies. This contractor also calibrates other equipment used by the Air Force for clinical chemistry, urinalysis, and hematological services. When necessary, THRU SOPs reference USAF SOPs in equipment calibration.

GLP Section 792.81. Postmortem and Microscopic Diagnosis

NSI-ES personnel conduct necropsies and prepare tissues for necessary histologic examination by USAF pathologists. Postmortem gross and microscopic diagnoses, preparation of histopathology incidence reports, and archiving of pathology specimens are all conducted by USAF personnel. Study protocols include USAF SOPs by reference, and they delineate areas of responsibility for study completion.

GLP Section 792.90. Animal Receiving

This is another area in which considerable cooperation exists for study completion. Health status evaluation and quarantine of newly received animals is performed by USAF personnel. There is significant interaction between the technical manager of the study, the THRU veterinary technicians, and the Veterinary Services Division of the USAF. THRU SOPs define these interactions and delineate functional responsibilities.

The GLP program has been designed for quarterly performance audits. Listed below are procedure audits conducted this year.

TD Number	Test Phase
USN 0-007	Dermal LD_{50}: Limit Test
USN 0-026	Primary Irritation Screen: skin irritation skin sensitization eye irritation inhalation LC_{50}: Limit Test oral LD_{50}: Limit Test

THB 0-004	Specimen Sampling: Pharmacokinetic inhalation LC_{50}

The GLP program was also structured so that a comprehensive data audit could be conducted at the completion of each study. The report and data audits conducted this year are listed below.

TD Number	Report Title
USN 0-015	Evaluation of the Sensitization and Acute Skin Irritation Potential of Navy Watch Cap Samples
	Evaluation of the Eye and Dermal Irritation and Dermal Toxicity of Acryloid 953 and TC9596A
THB 0-011	Acute Toxicity Testing for Selected Groundwater Contaminants: Chemical Analysis
	Evaluation of the Inhalation and Skin Absorption Kinetics of a Cyclotriphosphazene Based Hydraulic Fluid: Chemical Analysis
	A Dermal Kinetic Study of Dimethylmethylphosphonate
	Absorption and Metabolism of Chlorotrifluoroethylene Oligomer After Oral, Dermal and Inhalation Exposure: Chemical Analysis
TH 0-009	Evaluation of the Acute Toxicity of Process Chemicals Associated with the Production of QL: Chemical Analysis of LT
	The Toxicological Evaluation of Polyalphaolefin Hydraulic Fluids

APPENDIX C

HEALTH AND SAFETY PROGRAMS

The safety and health of the personnel conducting toxicology studies at the THRU are of paramount importance. Consequently, during the first year, major facilities modifications were conducted, new equipment was upgraded, and new program direction was established. A THRU Safety Program was initiated.

A Safety Committee was established to ensure a safe work environment. Mr. Don Brade, the THRU Safety Officer, was appointed Chairman of the Committee. Ms. Lois Doncaster represents the Administrative Group, Mr. Richard Godfrey represents the Toxicology Group, and Ms. Vicki Harris is the representative of the Engineering and Chemistry Group. Monthly meetings of the Safety Committee are periodically attended by the USAF Safety Monitor to ensure coordination between NSI-ES and the Air Force in program policies.

Operational activities of the Safety Committee consist of monthly tours and inspections of the different sections of the THRU. These are designed to identify and assist employees in solving safety-related problems. Other activities include establishing operating procedures and safety and emergency procedures.

APPENDIX D

ANIMAL TECHNICIAN TRAINING PROGRAM

The number of NSI-ES animal technicians who have become AALAS certified are listed below.

- Laboratory Animal Technologists, six
- Laboratory Animal Technicians, four
- Assistant Animal Technicians, two

The outline of the AALAS course was described in detail in a previous THRU report (MacEwen and Vernot, 1975).

The animal technicians are responsible for the care, feeding, and associated tasks of all research animals housed in AAMRL facilities. This includes all animals housed in Buildings 79, 429, 433, 838, and 839 in Area B, Wright-Patterson Air Force Base. Duties encompass animal care during animal quarantine, holding and pre- and postexposure periods.

Education of the entire group of technicians has included necropsy training as well as new or refresher (primate care) programs. Courses completed by newly hired technicians include the Good Laboratory Practices training and the Biotech Series, which covered small animal bleeding techniques, oral dosing, handling, and restraint of laboratory animals. Training programs currently in progress include a practical training course that teaches techniques that all technicians should be able to perform.

Advanced training programs will also be provided for the technicians, including the laboratory animal medicine technologist training course on video-tapes. The Purina Animal Care course is primarily a self-study course to be completed under the direction of a supervisor. Tests covering the four main sections of the course will be given at the end of each section. Inhalation studies currently being planned for the Thomas Domes have brought about a need for formal training in that area. A training schedule for dome entry is attached.

A complete list of the programs and training utilized by the technician group is given below.

- Purina Animal Care
- Biotech Small Animal Series
- Good Laboratory Practices
- Practical Training List
- Advanced Practical Training List
- Toxic Hazards Research Unit Animal Care
- Laboratory Animal Medicine and Science Audiotutorial Series
- Dome Flight Training
- Stark/McBride Technologist Correspondence Course
- Medical Terminology
- Toxic Hazards Research Unit Parapathologist Video Tapes
- Laboratory Animal Technologist Video Tapes

REFERENCES

MacEwen, J. D., and E. H. Vernot. 1975. Toxic Hazards Research Unit Annual Technical Report: AMRL-TR-75-57, (ADA-019456) Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, OH.

APPENDIX E

OPEN LITERATURE PUBLICATIONS

This Appendix provides the titles and abstracts of all papers published by NSI-ES/THRU scientists during the current contract year. The THRU scientific staff collaborates with outside toxicologists, a fact that is reflected in the diversity of published subject matter. In addition, during the first contract year, this publication list includes work carried out at staff members' previous institutions.

Yeh, H. C., G. J. Newton, E. B. Barr, R. L. Carpenter, and C. H. Hobbs. Studies of the temporal and spatial distribution of aerosols in multi-tiered inhalation exposure chambers. *American Industrial Hygiene Association Journal*, Volume 47, pages 540-545, 1986.

Two multitiered whole-body inhalation exposure chambers with nominal volumes of 1 m^3 (H-1000 chamber) and 2 m^3 (H-2000 chamber) were evaluated for their performance in terms of the temporal and spatial distribution of test aerosols within the chamber. Parameters investigated included chamber type, single-chamber-single-aerosol generator versus two-chamber-single-aerosol generator systems, chamber air supply and exhaust systems, particle size, and aerosol diluter type. Results indicated that: 1) particle size has an effect on chamber aerosol concentration distribution, with the larger particle resulting in a higher variation; 2) the single-chamber-single-generator system is more stable than the two-chamber-single-generator system; 3) the H-2000 chamber has a lower aerosol spatial variability than the H-1000 chamber; and 4) the aerosol distribution within the chamber could be improved with the use of a newly designed diluter.

B. H. Gray, M. Porvaznik, C. D. Flemming and L. H. Lee. Inhibition of tributyltin mediated hemolysis by mercapto compounds. *Journal of Applied Toxicology*, Volume 6, Number 5, pages 336-370, 1986.

Hydrophobic tributyltin (TBT) compounds at concentrations greater than 10 μm caused hemolysis of human erythrocytes and formed structures in plasma membranes. The mercapto compounds, B-mercaptoethanol (BMER), 2,3-dimercaptopropanol (BAL), 2,3-dimercapto-1-propane sulfonate (DMPS), DL-dithiothreitol (DTT), and meso-2,3-dimercaptosuccinic acid (DMSA) were examined for their ability to inhibit TBT-mediated hemolysis. The relative order of effectiveness for inhibition of TBT-mediated hemolysis was BAL>DTT>DMSA>DMPS>BMER. A fourfold excess of BAL over TBT prevented hemolysis for 4 h, and addition of BAL 0.5 h after TBT reduced the rate of hemolysis. The number of membrane-associated TBT aggregates observed per cell profile decreased as the BAL concentration increased from 0 to 100 μm. However, the mean diameter of TBT aggregates nearly doubled in erythrocyte suspensions at 100 μm BAL. Reactions of dimercapto

compounds with lipophilic TBT aggregates may depend on their relative lipid solubilities. Also, conversion of the weak Lewis acid, TBT, from a four- to a five- or six-coordinate tin adduct by the dimercapto Lewis bases used could also be a factor of the slowing hemolysis rates.

B. H. Gray, M. Porvaznik, C. D. Flemming and L. H. Lee. Organotin-induced hemolysis, shape transformation and intramembranous aggregates in human erythrocytes. *Cell Biology and Toxicology* (in press).

Organotin compounds examined in this study exhibited a relative order of potency for induction of *in vitro* hemolysis in human erythrocytes as follows: TBT>TPT>TTBT>TPhT>TET >DBT>stannous chloride>TMT = MBT. All of the organotin compounds examined induced erythrocyte shape transformation from the normal discocyte to either an echinocytic or stomatocytic form. In addition, TPhT, TTBT, and TET each elicited stomatocyte formation at sufficiently high concentrations. Select organotin compounds also formed tin-containing aggregates within the plasma membrane. The relative order of effectiveness for organotin induction of intramembranous aggregates was TBT>TPT>TTBT>TET, based upon the lowest concentration at which aggregates were observed. These results support the previously suggested theory that organotins are membrane effectors because of their comparatively high hydrophobic, lipid partitioning properties. The relatively lipophilic compound, TPhT, appeared to be anomalous because it did not readily promote hemolysis or induce the formation of intramembranous aggregates in human erythrocytes. A log-linear statistical model demonstrated association of hemolysis with both TBT aggregate formation and shape transformation. Select organotin compounds should be useful probes in membrane studies because of their numerous effects.

Cibulas, W., Jr., C. G. Murlas, M. L. Miller, A. Vinegar, D. J. Schmidt, R. T. McKay, I. L. Bernstein, and S. M. Brooks. Toluene diisocyanate-induced airway hyperactivity and pathology in the guinea pig. *Journal of Allergy Clinical Immunology*, Volume 77, pages 828-834, 1986. Work performed at University of Cincinnati and supported by grant Numbers ES-02847 and ES-00159 from the National Institutes of Health.

We assessed the nature and progression of airway mucosal disease and histaminic reactivity in English short-haired guinea pigs at 2, 24, 72, 168, and 504 h after toluene diisocyanate (TDI) exposure (4 h of 3 ppm of TDI for 5 consecutive days). To also determine whether TDI-specific, IgE-like antibodies developed in TDI-exposed animals, passive cutaneous anaphylaxis testing was done 28 days after TDI. Bronchial reactivity was determined serially by measuring specific airway conductance as a function of increasing doses of aerosolized histamine in six exposed and three control animals studied intact and unanesthetized. The remaining 10 exposed and 10 control guinea pigs were sacrificed in groups of 2 at each time point to obtain airway tissue for light microscopic

examination. We found that airway hyperreactivity to histamine occurred after TID in all animals tested. It was maximal 2 h after the 5-day exposure and remitted by 72 h. In addition, marked airway obstruction occurred after TID that persisted for at least 168 h. There were dramatic signs of airway mucosal damage associated with the bronchial hyperreactivity that included substantial decreases in epithelial cilia, mucin content, and most cells, as well as squamous metaplasia, numerous mitotic figures, and a prominent polymorphonuclear leukocytic infiltrate. Passive cutaneous anaphylaxis tests in exposed animals were negative. Our results suggest that TDI-induced bronchial hyperreactivity may be related to airway mucosal injury and inflammation.

Miller, M. L., A. Andringa, A. Vinegar, W. D. Adams, W. Cibulas, Jr., and S. M. Brooks. Morphology of tracheal and bronchial epithelium and type II cells of the peripheral lung of the guinea pig after inhalation of toluene diisocyanate vapors. *Experimental Lung Research*, Volume II, pp. 145-163, 1986. Work performed at University of Cincinnati and supported by grant Numbers ES-02847 and ES-00159 from the National Institutes of Health.

Toluene diisocyante (TDI), a polymerizing agent used in production of plastics, can cause airway disease in some exposed individuals. Using guinea pigs as a model, the response of the airways and the type II cells of the peripheral lung was monitored morphologically and morphometrically after exposure to TDI vapors at 30 ppb, 260 ppb, and 3100 ppb. The two low doses of TDI caused little change in airway epithelium. There was no gross inflammatory cell infiltrate; however, surface infoldings and intracellular ciliated cysts increased in numbers.

Animals exposed to 3100 ppb TDI 4 h/day for 5 days sustained considerable damage to the epithelium, and stratified non-keratinizing cells lined the airways until one week after exposure. Polymorphonuclear leukocytes were present in the early period after exposure. Increased numbers of eosinophils were present between one and two weeks following exposure. Mitoses in the epithelium were common during recovery.

In the peripheral lung, though a modest subjective increase in the number of type II cells was seen after 3100 ppb TDI, the volume density of type II cells and organellar components (lamellar bodies, mitochondria, cisternal bodies) did not change significantly after any exposure level of TDI.

Baughman, R. P., C. K. Kim, A. Vinegar, D. E. Hendricks, D. J. Schmidt, and W. E. Bullock. The pathogenesis of experimental pulmonary histoplasmosis: Correlative studies of histopathology, bronchoalveolar lavage, and respiratory function. *American Review Respiratory Diseases*, Volume 134, pages 771-776, 1986. Work performed at University of Cincinnati and supported by grant Number AI-17339 from the National Institutes of Health.

A murine model of acute pulmonary histoplasmosis was employed to study the pathogenesis of the disease process by means of histopathology, bronchoalveolar lavage, and respiratory function

tests. These studies were performed on C67BL/6 mice from 8 h to 8 wk following intranasal inoculation of 10^5 yeast forms of *H. capsulatum* and on age-matched controls that received saline only. At week 1, the histopathology was characterized by inflammation consisting of polymorphonuclear (PMN) subacute leukocytes, lymphocytes and macrophages that infiltrated the interstitium around small bronchioles and adjacent alveoli. At weeks 2 and 4, the infiltrates were comprised predominantly of lymphocytes and macrophages. Aggregates of lymphoid cells were prominent along the bronchial tree and in perivascular distribution. Those in close contact with bronchiolar epithelium resembled hyperplastic bronchus associated lymphoid tissue. Quantitative studies of cells in the BAL fluid revealed a large influx of polymorphonuclear leukocytes at week 1 with return to normal range by week 2. At this time there was a significant ($p<.02$) increase in lymphocytes that persisted through week 8 although histopathologic changes were minimal in lung at this time. A significant decrease in the DLCO/TLC at week 2 in association with a normal vital capacity indicated impairment of respiratory function secondary to the alveolitis induced by *H. capsulatum* infection rather than a reduction of lung volume.

This model offers promise for additional correlative studies of lymphocyte subsets in lung tissue and alveolar spaces as well as of the functions subserved by these respective populations.

Conolly, R. B., Cramer, J. L., and Andersen, M. E. A physiologically based model for rat hepatic glutathione (GSH): Its circadian oscillation and interaction with halogenated hydrocarbons. *The Pharmacologist*, Volume 128, page 211, 1986.

In the normally fed rat, hepatic GSH levels oscillate over a 24-h period with a maximum (7100 μM) at about 10 AM and a minimum (4300 μM) at about 10 PM. In fasted rats there is little, if any, oscillation and the mean GSH level is 3800 μM (Jaeger *et al.*, *Res. Commun. Chem. Pathol. Pharmacol.* 6:465-471, 1973). Our model simulates hepatic GSH levels of fed and fasted rats. The model describes eating behavior, uptake from the GI tract of the GSH precursors cysteine and methionine (-SH), hepatic synthesis of GSH and its feedback inhibition by GSH, saturable secretion of GSH from the liver, first-order loss of -SH and GSH from the liver, and recycling of -SH to the liver from other tissues. The model can be used to study how time of day and pre-exposure fasting affect GSH-dependent pharmacodynamic processes for compounds such as 1,1-dichloroethylene and 1,2-dichloroethane (EDC). For example, when 4 h inhalation exposure to 100 ppm EDC is simulated, the model predicts significantly greater production of the DNA-damaging conjugate of EDC with GSH when exposure starts at 10 AM rather than 10 PM. (This work was supported in part by U.S. EPA CR812556. It does not necessarily reflect EPA policy.)

Lubawy, W. C., Culpepper, B. T., and Valentovic, M. A. Alterations in prostacyclin and thromboxane formation by chronic cigarette smoke exposure: Temporal relationships and whole smoke vs. gas phase. *Journal of Applied Toxicology*, Volume 6, pages 77-80, 1986.

Chronic cigarette smoke exposure *in vivo* causes decreased conversion of [^{14}C]arachidonic acid (AA) to prostacyclin (PGI_2) by isolated aortic tissue and increased conversion to thromboxane (TXA_2) by isolated platelets from rats. Alterations in the PGI_2/TXA_2 balance may be part of the mechanism through which smoking increases the risk of cardiovascular disease. To study the influence of smoke exposure duration on this response, male rats were exposed daily to 10 puffs of freshly generated cigarette smoke. Animals were killed after 1, 4, 14, 28 and 47 days of smoke exposure and 3, 7, 14 and 28 days after cessation of the 57-day of smoke-exposure regimen. Elevated carboxyhemoglobin levels during the smoke-exposure sessions verified smoke (gas phase) inhalation. Statistically significant alterations in prostacyclin synthesis preceded those of thromboxane. A decrease of 20-25% ($p<0.05$) in PGI_2 production from [^{14}C]AA in isolated aortic tissue was found beginning 28 days after smoke was initiated and quickly rebounded when smoke exposure was terminated. Increased production of TXA_2 from [^{14}C]AA by isolated platelets became statistically significant ($p<0.05$) on the 57th day and returned to normal 7-14 days after cessation of smoke exposure.

To determine the effect of gas-phase constituents on the PGI_2/TXA_2 balance, a second series of experiments divided male and female Sprague-Dawley rats into sham, whole smoke and gas-phase groups. Gas phase was produced by passing whole smoke through a Cambridge filter to remove particulate matter. Percent COHb averaged 1.4 for sham, 7.8 for whole smoke and 9.4 for gas-phase groups. After 8 and 12 weeks of exposure, whole smoke decreased aortic PGI_2 and increased platelet TXA_2 both in male and in female rats. In contrast to whole smoke exposure, 8 or 12 weeks of exposure to gas-phase constituents failed to produce any statistically significant alteration in PGI_2 and TXA_2 formation.

It was concluded that changes in PGI_2 and TXA_2 formation occur readily following initiation or termination of chronic whole smoke exposure and that substances present in the particulate phase of smoke are essential to producing these changes. (Supported in part by University of Kentucky Tobacco and Health Research Institute Project Number 4B006.)

Lubawy, W. C., Culpepper, B. T., and Sherratt, A. J. Alterations in prostacyclin and thromboxane formation following chronic exposure to high and low nicotine cigarettes in rats. *Artery* (in press).

Chronic exposure to cigarette smoke causes an imbalance in both PGI_2 and TXA_2 production, which is believed to favor the development of atherosclerosis. Nicotine, a major constituent of smoke, has been shown to adversely alter arachidonic acid metabolism. To determine whether varying doses of nicotine in cigarettes would influence the extent of PGI_2/TXA_2 alterations, male

Sprague-Dawley rats were chronically exposed (7 days/wk/6 mo.) to smoke from University of Kentucky Reference cigarettes containing "low" nicotine (3A1), "high" nicotine (2R1) and "low" nicotine cigarettes spiked with enough nicotine to deliver amounts equivalent to the 2R1. COHb levels were monitored to confirm smoke inhalation. No differences in body weights were observed between treatment groups, but all were significantly lower than sham. Aortic PGI_2 formation was not significantly depressed by any of the treatment groups compared to shams, irrespective of nicotine content. In the rat smoking model, low-nicotine cigarettes do not appear to be an advantage over higher nicotine cigarettes. (Supported in part by University of Kentucky Project Number KTRB 5-41072.)

Sherratt, A. J., Culpepper, B. T., and Lubawy, W. C. Prostacyclin and thromboxane formation following chronic exposure to cigarette smoke condensate administered via osmotic pumps in rats. *The Pharmacologist*, Volume 28, page 183, 1986.

Chronic cigarette smoke decreased aortic PGI_2 and increased platelet TXA_2 production. In order to ascertain whether the particulate phase of whole smoke alone could cause these changes, rats were administered smoke condensate in propylene glycol for 56 days via 2 Alzet (2ML4) osmotic pumps. Pumps containing vehicle, low dose (150 ug/hr) or high dose (300 ug/hr) condensate were implanted s.c., dorsal to the thoracic vertebrae in male Sprague-Dawley rats. Three-quarters of the condensate treated rats developed fibrin cysts encapsulating the pumps. Cysts were not seen in vehicle-treated rats. Residual pump contents were weighed and analyzed by GLC to ensure condensate delivery. No significant difference in weight gain patterns between sham operated and treatment groups was observed. Vehicle had no effect on aortic PGI_2 or platelet TXA_2 formation compared to sham. Low-dose condensate was without effect on PGI_2/TXA_2 formation. In high-dose condensate treated rats, PGI_2 and TXA_2 formations were 84 and 136%, respectively, of the vehicle control (n.s.). Pump encapsulation may be a limiting factor in the administration of whole smoke condensate. (Supported in part by University of Kentucky Project Number KTRB 5-41072.)

Kutzman, R. S., Drew, R. T., and Shiotsuka, R. N. Pulmonary changes resulting from subchronic exposure to cadmium chloride aerosol. *Journal of Toxicology and Environmental Health*, Volume 17, pages 175-189, 1986.

Fischer 344 rats were exposed to 0.0, 0.3, 1.0, or 2.0 mg Cd/m^3 as $CdCl_2$ aerosol for 6 h/d, 5 d/wk, for 62 exposure days. Exposure to 2.0 mg Cd/m^3 resulted in rapid weight loss, and all of the animals died within the first 45 days of exposure. As a group, female rats survived significantly longer than the males. Exposure to Cd resulted in dose-dependent increases in lung weight. The increased weight was the result of additional tissue mass rather than edema. Both connective-tissue components, elastin and collagen, were significantly increased in the 1.0 mg/m^3 group when these

components were expressed on the basis of dry weight. Dose-dependent changes at the terminal bronchioles consisted of hyperplasia and flattening of type II cells, inflammation, and the proliferation of fibroblasts. Exposure to Cd also resulted in the development of intralymphatic microgranulomas in the perivascular and peribronchiolar lymphoid tissues.

APPENDIX F

PRESENTATIONS

Carpenter, R.L., E.C. Kimmel, and C.D. Flemming. 1986. Toxicant Distribution Measurements in the Thomas Domes. Presented at the 18th Aerosol Technology Meeting, Pacific Grove, CA, August 17-30.

Gray, B.H., M. Porvaznik, N.J. Fouts, S.C. Cummings, L.H. Lee, and C.D. Flemming. 1986. Tri-n-butyltin: A Membrane Toxicant. Presented at a meeting in Santa Barbara, CA , January 27-31.

Gray, B.H., M. Porvaznik, C.D. Flemming, and L.H. Lee. 1986. Tri-n-butylin Aggregates and Membrane Cytotoxicity in Human Erythrocytes. Presented at OCEANS.

Conolly, R.B., J.L. Cramer, and M.E. Andersen. 1986. A Physiologically Based Model for Rat Hepatic Glutathione (GSH) and Its Interaction with Halogenated Hydrocarbons. Presented at the Joint Meeting American Society for Pharmacology and Experimental Therapeutics and Society of Toxicology, Baltimore, MD, August 17-21.

Conolly, R.B., R.H. Reitz, and M.E. Andersen. 1986. Mutation Accumulation after Repeated Cycles of Cell Death and Regeneration: A Biologically Based Mathematical Model of Chronic Exposure to a Cytotoxicant. Presented at the National Institute of Environmental Health Sciences, National Toxicology Program, National Institute of Occupational Safety and Health Conference: Tumor Promoters: Biological Approaches for Mechanistic Studies and Assay Systems. National Institute of Environmental Health Sciences, Research Triangle Park, NC, September 8-10.

Conolly, R.B. 1986. Application of Mathematical Models to Volatile Toxicant Pharmacokinetics and the Relationship of Cytotoxicity to Mutation Accumulation. Presented at a seminar given to the Department of Environmental and Community Medicine, Robert Wood Johnson Medical School, University of Medicine & Dentistry of New Jersey, Piscataway, NJ, October 1.

Conolly, R.B., R.H. Reitz, and M.E. Andersen. 1986. Mutation Accumulation: A Biologically Based Mathematical Model of Chronic Cytotoxicant Exposure. National Academy of Sciences, National Research Council, Board on Environmental Studies and Toxicology, Safe Drinking Water Committee, Subcommittee on Pharmacokinetics Workshop: Pharmacokinetics in Risk Assessment. National Academy of Sciences, Washington, DC, October 7-9.

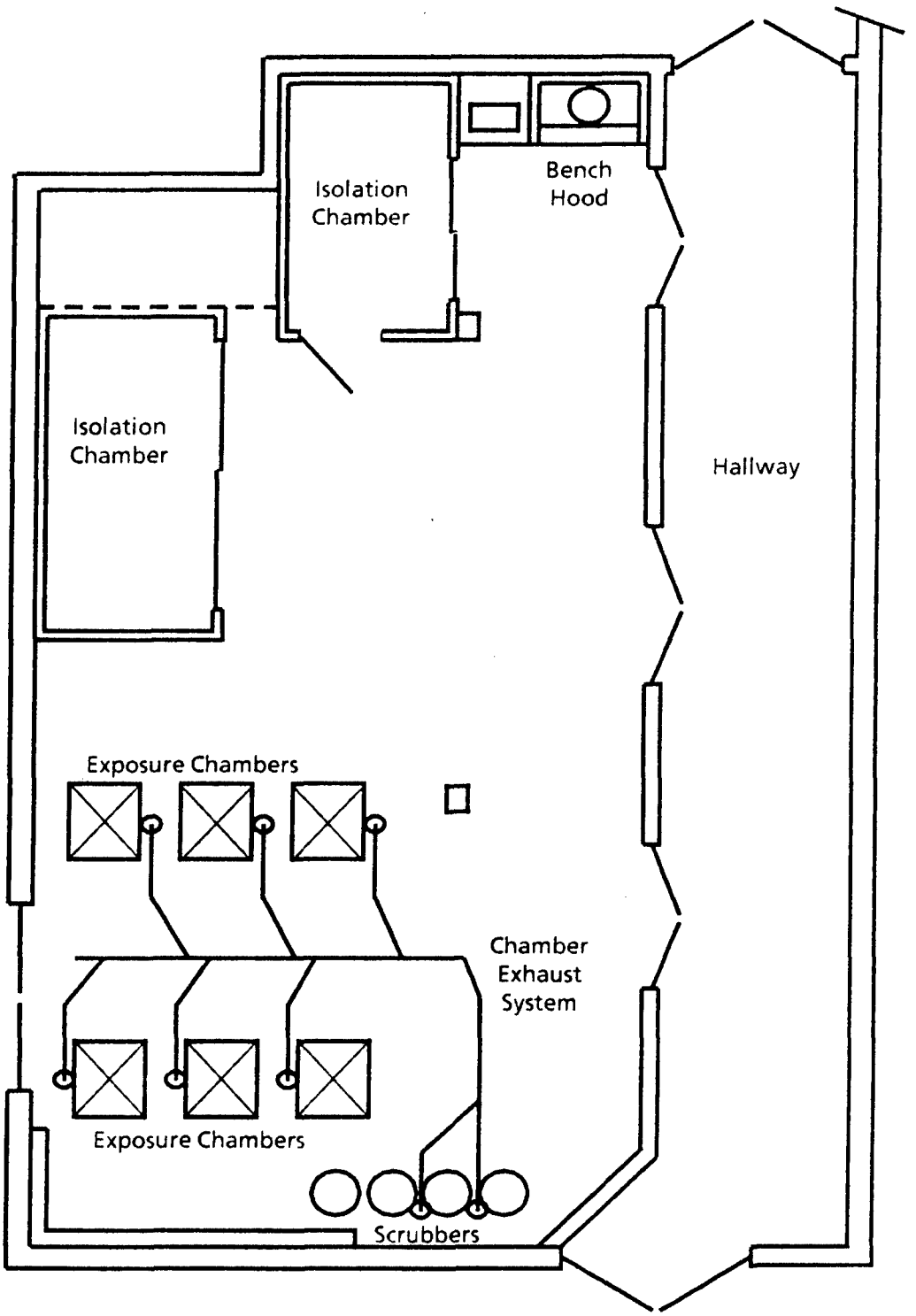
APPENDIX G

VISITING SCIENTIST/SEMINAR TITLES

6/3/86	Dr. P. V. Shah; Skin Penetration of Pesticides in Rats; Host - Dr. R. S. Kutzman
6/19/86	Drs. Vernon Steele and Dr. Eugene Elmore; NSI's *In Vitro* Toxicology Capability; Host - Dr. R. S. Kutzman
6/19/86	Dr. David Clark; Analysis of Products from the Pyrolysis of Sarin; Host - Dr. R. L. Carpenter
7/29/86	Dr. James Hern; Current Applications of Short-Term Toxicity Testing - Development of Inductively Coupled Plasma Atomic Emission Spectroscopy for Biological and Environmental Analysis; Host - Dr. R. L. Carpenter
7/30/86	Dr. Russel Wiener; Current Applications of Short-Term Toxicity Testing - Particle Losses in Sampling Probe Inlet at Low Air Velocities; Host - Dr. R. L. Carpenter
8/11/86	Dr. Moheb Makary; Hydroxylation of the Esophageal Carcinogen Methyl-*n*-Amylnitrosamine (MNAN) by the Rat Esophagus and Related Tissues; Host - Dr. R. B. Conolly
8/12/86	Dr. Jean Cramer; DNA Damage Model; Host - Dr. R. B. Conolly
8/13/86	Dr. Patricia Conway; Long-Term *In Vitro* Operation of Enzyme Based Glucose Sensors; Host - Dr. R. B. Conolly
8/14/86	Dr. Gary Ginsberg; Acetaminophen Hepatotoxicity: Covalent Binding to and Effects on Hepatic Organelles; Host - Dr. R. B. Conolly.
9/11/86	Dr. William Amos; Round Table Discussion; Host - Dr. R. L. Carpenter
9/26/86	Dr. Robert Vanderslice; Limitations of Pharmacokinetic Models for the Assessment of Tetrachloroethylene Cancer Risk; Host - Dr. R. B. Conolly

APPENDIX H

GENERAL TOXICOLOGY LABORATORY FLOOR PLAN



☆ U.S. GOVERNMENT PRINTING OFFICE: 1987 - 748-001/40238